Phase separation in fungi

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Abstract

Phase separation, in which macromolecules partition into a concentrated phase that is immiscible with a dilute phase, is involved with fundamental cellular processes across the tree of life. We review the principles of phase separation and highlight how it impacts diverse processes in the fungal kingdom. These include the regulation of autophagy, cell signaling pathways, transcriptional circuits and the establishment of asymmetry in fungal cells. We describe examples of stable, phase-separated assemblies including membrane-less organelles (MLOs) such as the nucleolus, as well as transient condensates that also arise through phase separation and enable cells to rapidly and reversibly respond to important environmental cues. We showcase how research into phase separation in model yeasts, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* in conjunction with that in plant and human fungal pathogens, such as *Ashbya gossypii* and *Candida albicans,* is continuing to enrich our understanding of fundamental molecular processes.

INTRODUCTION

Organelles are often bound by a lipid membrane that separates internal components from the surrounding environment, but over the past decade multiple compartments have been identified in which macromolecules are concentrated in the absence of a phospholipid membrane. Such membrane-less organelles (MLOs) form by phase separation which involves de-mixing of a super-saturated solution into a dense phase that exists together with a more dilute surrounding phase[1]. Many MLOs have liquid-like behaviour consistent with liquid-liquid phase separation (LLPS), and their components can undergo rapid exchange with the surrounding environment[2,3]. The physics of polymer systems undergoing phase separation have been well studied through thermodynamic models such as the Flory-Huggins theory[4]. When a polymer is mixed with a solvent, phase separation can occur above a crucial concentration that depends on environmental factors (temperature, osmolarity, and pH) that alter the effective favourability (free energy) of polymer-polymer interactions. MLOs that form via phase separation take several forms, including liquid-like assemblies (via LLPS), semi-solid gels (with viscoelastic behavior) and rigid fibrillar aggregates, and are associated with diverse cellular processes throughout the fungal kingdom. A primer on the molecular forces underlying phase separation is provided in BOX 1.

Here, we review the diverse roles of biomolecular condensates in model and pathogenic fungi, including their function in autophagy, cytoskeletal organization and cell polarity, transcriptional regulation of cell fate, and sensing and responding to the cellular environment.

Stress-induced bodies formed by phase separation

Stress granules (SGs) and processing (P) bodies are dynamic MLOs that assemble on mRNAs stalled in translation (FIG. 1). Different SGs can form depending on the stress, which leads to the release of mRNAs encoding heat shock and chaperone factors thereby enabling their translation and stress adaptation[5]. P bodies are constitutively present in the cytoplasm and share components with

SGs, but do not include translation initiation factors and instead sequester proteins associated with mRNA decay[6]. SGs and P bodies therefore perform distinct functions, and these MLOs can also be found docked against one another with mRNAs moving between them[7]. In *S. cerevisiae*, increased temperature or depletion of specific nutrients triggers SG formation and is driven by high concentrations of IDRs forming on mRNPs following stalled translation[8]. This causes the assembly of condensates that then mature into a less dynamic, stable core that is surrounded by a liquid shell[8]. Both yeast and mammalian SGs share a similar architecture, although a more extensive solid core in yeast SGs makes them less dynamic overall[8,9].

S. cerevisiae poly(A)-binding protein Pab1 is a canonical SG factor that contains four RNA recognition motifs (RRMs) as well as a proline-rich disordered domain (P domain)[5,10]. Pab1 phase separates *in vitro* to form gels in response to physiological cues such as a reduction in pH or heat shock[5]. RRMs promote Pab1 phase separation through electrostatic interactions, while hydrophobic interactions between P domains enhance this process[5]. A second *S. cerevisiae* SG protein, Pub1 (poly(U)-binding protein), is similar to Pab1 in that RRMs drive self-assembly while IDRs modify condensate properties[11]. Notably, Pub1 condensates show distinct material states depending on the stress; purified Pub1 forms reversible, gel-like condensates in response to low pH whereas more solid-like structures arise following heat shock, replicating observations with Pub1-containing condensates in cells[11]. Interestingly, only the more solid, thermally-induced condensates require the Hsp104 chaperone for dissolution[11] (FIG. 1), indicating differences in the structural architecture of these condensates. Heat-induced condensates formed by Pab1 are similarly dispersed by an active disaggregation system consisting of Hsp40/Hsp70/Hsp104 chaperones which therefore helps cells recover from heat shock[12].

P bodies function in RNA metabolism including mRNA storage/decay, and are also induced by stress[13]. These bodies are archetypal condensates with liquid-like properties both in *S. cerevisiae* and in mammals, and several purified P body factors have been shown to undergo LLPS *in vitro*[9,14,15].

Multivalency in P bodies is achieved by interactions between folded protein domains, IDRs, and RNA[14]. The most highly enriched P body proteins in *S. cerevisiae* (Dcp2, Pat1, and Edc3) partition cooperatively into these bodies as well as promote P body assembly[16] (FIG. 1). Importantly, these studies suggest that only a few factors need to evolve LLPS capacity in order for MLOs to form[16]. In heavily stressed cells, the liquidity of P bodies is maintained by Hsp104 and loss of this activity results in P body proteins entering into SGs, further highlighting how disaggregases determine the behavior of stress-induced MLOs in the cell[9].

A third class of stress-induced condensate in *S. cerevisiae* is the glycolytic (G) body, which involves glycolytic enzymes assembling into gel-like condensates during hypoxia[17,18]. G bodies enable growth under hypoxic conditions when oxidative phosphorylation is unavailable, most likely by concentrating glycolytic enzymes within condensates and promoting glucose consumption[18]. G body formation involves multivalent protein-protein and protein-RNA interactions and, as with other MLOs, RNA may act as a scaffold for development of these bodies[17]. Additional factors recruited to G bodies include Hsp70 chaperones (Ssa1/Ssa2) and the AMP-activated protein kinase Snf1p, with the latter necessary for G body formation[18]. Analogous G-like bodies are present in human hepatocarcinoma cells under hypoxic stress and where RNA enables the formation of these metabolic bodies[18], suggesting conservation across eukaryotes.

Changes to the cellular environment that drive phase separation

Upon depletion of energy, *S. cerevisiae* and *S. pombe* yeast cells enter into a state of dormancy associated with cytoplasmic acidification[19,20]. The drop in intracellular pH occurs because culture medium is acidic whereas the intracellular pH is neutral, and cells must expend energy to maintain this pH difference[21,22]. Energy depletion and intracellular acidification cause a number of cytoplasmic proteins to assemble into microscopically visible foci or filaments[19,23]. Under these conditions, the yeast cytoplasm also transitions from its normal fluid state to a more solid-like state, which may be

because much of the yeast proteome becomes less soluble and forms higher order assemblies[19,20,24]. This response is not unique to yeast cells as bacterial cells similarly respond to glucose starvation by transitioning from a glassy-liquid to a solid-like state, suggesting that these transitions are conserved and promote adaptation to stress[20,25].

The *S. cerevisiae* translation termination factor Sup35, long studied for its ability to form a heritable prion, also forms condensates upon a drop in intracellular pH[26]. Sup35 consists of an N-terminal prion-like domain (PrLD; see BOX 1), a negatively charged middle (M) domain, and a C-terminal GTPase domain that catalyzes translation termination. Franzmann *et al*. showed that the M-domain acts as a pH sensor and causes Sup35 to form gel-like droplets following a stress-induced pH reduction, and that droplets redissolve upon restoration of pH, both *in vitro* and in cells[26]. In contrast, the Sup35 C-terminal domain forms irreversible aggregates during stress when expressed alone, as the PrLD is necessary for preventing aggregate formation. Reversible gel formation appears to be the ancestral role of Sup35, as the *S. pombe* ortholog cannot propagate as a prion but shares the ability to form stress-induced condensates[26]. Thus, most PrLDs (*S. cerevisiae* contains >200 proteins with such domains) likely function to modulate phase separation or protein solubility rather than act as prions, with the latter being the exception rather than the rule[27]. Debate continues as to the relative importance of *S. cerevisiae* Sup35 in forming condensates versus the prion state, with both roles being potentially beneficial to this species[28].

Related studies have demonstrated that increased macromolecular crowding can promote phase separation events. Crowding agents such as polyethylene glycol have been extensively used to increase effective protein concentrations and promote phase separation *in vitro*[29]. To examine how macromolecular crowding influences behavior within cells, Delarue *et al.* performed microscopic tracking of genetically encoded multimeric (GEM) nanoparticles[30]. GEMs consist of a fluorescent molecule fused to a scaffold which multimerizes into particles of a defined shape and size. When expressed in *S. cerevisiae* cells, the motility of GEMs was reduced in the relatively crowded nucleus

129 compared to that in the cytoplasm[31] (FIG. 2a). Using this system, increased mTORC1 activity was
130 shown to increase macromolecular crowding due to an increase in ribosome number[30] (FIG. 2b).
131 Ribosomes are a major cellular component (~200,000 ribosomes are present per yeast cell) and occupy
132 ~20% of the cytosolic volume; an increase in the number of ribosomes therefore increased both
133 macromolecular crowding and phase separation of a cytosolic protein[30]. These experiments establish
134 close links between ribosome concentrations, macromolecular crowding and phase separation in yeast,
135 with similar results obtained in a human cell line[30]. A recent study showed that increased
136 macromolecular crowding also occurs in energy-starved *S. cerevisiae* cells due to a reduction in cell
137 size, which in turn supports the formation of MLOs[24].
138 Taken together, these studies reveal that starvation can drive phase separation and
139 oligomerization of cellular factors due to both changes in intracellular pH and increased molecular
140 crowding. Recent experiments further show that hyperosmotic stress can also drive the formation of
141 intracellular protein foci (OSF; osmotic shock foci) which may represent liquid droplets formed upon
142 increased intracellular crowding[32]. Given that biocondensates arise in response to multiple stresses, it
143 is important to note that protein constituents can be shared between different stress-induced
144 condensates. For example, Sup35 and the SG protein Pab1 partially colocalize in pH-stressed cells but
145 not in starvation-stressed cells[26]. A key ongoing research question is to therefore determine what
146 controls the targeting of molecules to different stress-induced condensates in the cell.
147
148 ***The cytoskeleton, cell polarity and control of nuclear division***
149 The cytoskeleton is a dynamic structure composed of filaments that undergo nucleation,
150 polymerization, and depolymerization. In both budding yeast and filamentous fungi, the polarisome
151 nucleates actin polymerization which involves the formin protein Bni1 together with nucleation
152 promoting factor (NPF) and the scaffold protein Spa2[33,34]. In *S. cerevisiae*, polarisome proteins
153 concentrate at the bud tip via LLPS to nucleate actin assembly while remaining in exchange with the

surrounding cytoplasm[34,35]. Xie *et al.* identified actin-interacting protein 5 (Aip5) as a factor that synergistically promotes actin assembly with Bni1[34]. Intriguingly, the N-terminal domain of Aip5 is an IDR that causes the formation of amorphous condensates *in vitro*, while addition of Spa2 to Aip5 assemblies turns these condensates into more dynamic, liquid-like droplets[34,35]. It is therefore envisaged that Spa2 prevents Aip5 aggregation during stress, which in turn enables condensate dissolution and supports the restart of actin assembly during recovery from stress[34,35] (FIG. 3a).

Ashbya gossypii is a filamentous fungus that is a plant pathogen and, like *S. cerevisiae*, belongs to the family *Saccharomycetaceae*. *A. gossypii* has emerged as an important model organism for studying cell polarity, filamentation and how asynchronous nuclear divisions occur within multinucleated cells. Remarkably, the protein Whi3 has been linked to the regulation of both cell polarity and the timing of nuclear divisions due to its ability to form RNA-dependent liquid droplets[36-38] (FIG. 3b). Whi3 establishes polarity at symmetry breaking points in the cell by forming condensates with Puf2 that incorporate *BNI1* and *SPA2* RNA transcripts[36,37]. In contrast, Whi3 droplets near nuclei contain *CLN3* mRNA (encoding for a G1 regulatory cyclin), and differences in the spatial distribution of this transcript determine the timing of the nuclear divisions in multinucleate cells[36,38]. Zhang *et al.* showed that the presence of different mRNAs results in distinct types of Whi3 condensates, from more liquid-like to more gel-like assemblies[36]. These results therefore provide a striking example of how RNA can impact the physical properties of biocondensates and can result in changes in their size, shape, viscosity, surface tension and composition[36,39] (also see BOX 2 for the central role of nucleic acids in promoting phase separation).

Autophagy

Autophagy is the organized breakdown of components by the lysosome, and provides energy and building blocks for cells to survive stress. This process involves formation of the Pre-Autophagosomal Structure (PAS) on the cytoplasmic face of the vacuolar membrane, which nucleates

assembly of a cup-shaped isolation membrane adjacent to the PAS that engulfs material in an autophagosome[40,41]. *S. cerevisiae* PAS formation involves five IDR-containing Atg (autophagy-related) proteins that co-assemble into the ATG1 complex (FIG. 4a)[40-42]. In starved cells, the TORC1 kinase is inactivated and Atg13 becomes dephosphorylated (by PP2C phosphatases) and establishes multivalent interactions with Atg17 to form highly liquid droplets[42,43]. Atg13 also interacts with the Vac8 membrane protein to anchor ATG1 droplets at the vacuolar membrane where they fuse to form one large condensate – the PAS[40,42]. Within the PAS, Atg1 is activated by autophosphorylation and phosphorylates Atg13, creating an equilibrium between phospho-Atg13 in the dilute phase and its unphosphorylated form in the condensed phase, which is important for maintenance of the PAS[42] (FIG. 4a). Formation of the cupped isolation membrane is not fully understood but is initiated by the recruitment of Atg9-containing vacuoles to the PAS via interactions with Atg13, and these vacuoles then fuse to generate the isolation membrane[44,45] (FIG. 4a). ATG proteins are conserved and phase separation is likely to play related roles in autophagy in multicellular organisms and yeast, although autophagosome formation is organized differently (both spatially and temporally) between these species[41].

A distinct form of autophagy, selective autophagy, targets specific organelles and biomolecules to the vacuole even under nutrient-rich conditions and also involves phase separation. *S. cerevisiae* Ape1 is the principle cargo of a form of selective autophagy termed the cytoplasm-to-vacuole targeting (Cvt) pathway[41,46]. Ape1 contains an N-terminal propeptide that can form a helical structure and self-assemble into dodecamers that then coalesce into semi-liquid droplets[46-48]. Atg19 acts as a receptor for Ape1 as it "floats" on the surface of Ape1 condensates and connects these condensates to Atg8/Atg21 (Fig. 4b)[46-48]. Through these interactions, a shape change in the isolation membrane enables it to form a phospholipid bilayer around Ape1 droplets and sequester them for degradation[47]. Changing a single amino acid in the Ape1 propeptide produced amorphous aggregates rather than gel-like droplets, and these hardened structures failed to interact with Atg19/Atg8 or undergo autophagy[47]. Similar results

have been observed in the targeting of *Caenorhabditis elegans* P granule proteins for autophagic degradation, where the gel-like state provides a suitable platform for engulfment by autophagosomal membranes[49]. These results highlight how the material properties of condensates are critical for determining the destination of cellular cargoes, both in fungi and higher eukaryotes.

Regulation of autophagy via sensing of reactive oxygen species

S. cerevisiae cells switched from a nutrient-rich medium to a minimal medium containing a non-fermentable carbon source undergo autophagy even in the continued presence of nitrogen, which can help cells maintain mitochondrial health during respiratory growth[50]. Tu and colleagues revealed that reactive oxygen species (ROS) produced by high mitochondrial dysfunction under these conditions are sensed by Pbp1, the yeast ortholog of mammalian ataxin-2[51,52]. Remarkably, Pbp1 is capable of undergoing phase separation in a redox-sensitive manner; this protein readily forms liquid- or gel-like droplets *in vitro* but these droplets dissolve upon the addition of hydrogen peroxide[51]. This mechanism involves oxidation of methionine residues within the Pbp1 LCR which therefore acts as a reversible readout of mitochondrial respiratory status[51]. Thus, Pbp1 condensates form during high respiratory growth and are poised to sense mitochondrial dysfunction and increased ROS levels, which in turn results in activation of TORC1 and inhibition of autophagy (as a means of adaptation to mitochondrial stress)[52,53]. These studies provide a striking example of how phase separation can be tuned by key physiological signals such as ROS. It was subsequently shown that human TDP-43 (involved in the formation of neuronal granules) similarly forms redox-sensitive condensates[54], highlighting how observations in *S. cerevisiae* led to the discovery of ROS sensing via phase separation in higher organisms.

Phase separation as a mechanism for sensing carbon dioxide

Cells have evolved sensitive mechanisms to detect changes in the levels of carbon dioxide

(CO_2). In fungi, CO_2 can affect multiple processes including mating, meiosis, phenotypic switching and filamentation[55]. Zhang *et al*. revealed that CO_2 sensing in the opportunistic human fungal pathogen *Candida albicans* involves condensates formed by Ptc2, a member of the PP2C family of phosphatases[56]. Here, a serine/threonine-rich region within the IDR of Ptc2 enabled this protein to undergo CO_2-induced phase separation, with CO_2 envisaged as a "molecular glue" that bridges interactions between Ptc2 molecules thereby stimulating condensate formation[56]. A related PP2C phosphatase from plants showed a similar ability to undergo CO_2-induced phase separation[56], indicating that this mechanism of environmental sensing (while uncovered in fungi) is likely conserved across diverse eukaryotic species[56].

Nuclear compartmentalization and heterochromatin

Phase separation plays a central role in the establishment of nuclear compartments such as the nucleolus which houses ribosome biogenesis and ribonucleoprotein assembly. This organelle is organized into three nested sub-compartments in higher eukaryotes; a core fibrillar center (FC) exists inside a dense fibrillar component (DFC), which itself resides within the granular component (GC)[57]. These compartments represent coexisting, immiscible liquid phases – this layered, multiphase architecture was reproduced *in vitro* as coincubation of a DFC protein with a GC protein generated multiphase droplets[57]. Here, IDRs within these proteins drive phase separation while RNA binding domains contribute to the immiscibility of the protein phases[57]. *S. cerevisiae* nucleoli have only two sub-compartments[58] and modeling suggests that ribosomal DNA (rDNA) is phase separated from bulk chromatin due to crosslinks between rDNA repeats[59], while nucleolar RNPs also undergo phase separation[60]. Indeed, the combination of rDNA phase separation and tethering of rDNA repeats to the nuclear envelope may explain the characteristic crescent shape of the yeast nucleolus[61].

Phase separation also controls the formation of transcriptionally repressed heterochromatin. Pioneering work on heterochromatin protein 1 (HP1) in higher eukaryotes showed that it can undergo

phase separation and recruit other heterochromatin-associated factors involved in transcriptional repression[62,63]. Constitutive heterochromatin is marked by trimethylation of lysine 9 on histone H3 (H3K9me3), and interactions between these marks and both HP1 chromodomains and SUV39H1 (which introduces H3K9me3 marks) are drivers of phase separation[64]. DNA polymers also contribute to the properties of heterochromatin resulting in stable structures that resist mechanical forces, while HP1 can exchange between condensate and non-condensate populations[65]. Swi6, the *S. pombe* homolog of HP1, similarly undergoes phase separation with reconstituted chromatin that contains H3K9me3 marks, suggesting parallels between constitutive heterochromatin formation in yeast and humans[64]. Related mechanisms may establish the formation of facultative heterochromatin in which polycomb proteins form phase-separated condensates and are associated with chromatin that is trimethylated on lysine 27 of histone H3 (H3K27me3)[66,67].

Phase separation, transcriptional activation and super-enhancer-like elements

Multiple studies have observed that transcription factors (TFs) can assemble into phase-separated complexes to activate gene expression[68-72]. Phase separation may occur preferentially at mammalian super-enhancers (SEs), where high concentrations of TFs, Mediator complex and RNA polymerase II (Pol II) span genomic regions >10 kb[69,70,72] (FIG. 5a,b). Weak multivalent interactions between TFs and coactivators together with structured interactions between TFs and DNA promote phase separation[70]. The sharply defined thresholds associated with condensate formation may underlie unique SE properties such as their hypersensitivity to changes in TF levels[69]. Moreover, the *de novo* assembly of a SE can be initiated by binding of a single TF to an enhancer, as exemplified by somatic mutations that cause MYB binding and subsequent TAL1 overexpression in T-cell acute lymphoblastic leukemia[73].

Mammalian SEs have been linked to transcriptional regulatory networks (TRNs) that define cell fate, where a core set of master TFs act in concert to control the expression of large numbers of

target genes[74,75]. Intriguingly, fungal TRNs are also regulated by sets of master TFs operating at super-sized regulatory regions ("SE-like elements"). Prominent examples of fungal TRNs include those regulating pseudohyphal formation in *S. cerevisiae*, the temperature response in *Histoplasma capsulatum*, biofilm formation and phenotypic switching in *C. albicans,* and the heat shock response that is conserved from fungi to humans[76-79]. The TRN regulating white-opaque phenotypic switching in *C. albicans* has been extensively analyzed and involves eight master TFs that act together at SE-like elements[80,81] (FIG. 5c,d). The master white-opaque TFs are often recruited to SE-like regions even in the absence of consensus DNA binding motifs, indicating they are likely recruited via protein-protein interactions. These regulatory TFs contain PrLDs that enable them to undergo phase separation *in vitro,* and mutations that block this process abolish their function, thereby linking phase separation to the regulation of this TRN[82]. Analysis of the heat shock response in *S. cerevisiae* similarly shows the presence of SE-like elements at which transcriptional condensates are expected to form[83,84]. Phase separation and SE-like regions are therefore implicated in transcriptional regulation in both fungi and mammals, as compared in TABLE 1.

Despite considerable interest in SEs it is currently unclear whether these elements are functionally distinct from regular enhancers, and the role of phase separation in transcription is also strongly debated[85,86]. Indeed, a recent study suggests that multivalent interactions can enable the formation of mammalian TF "hubs" that promote gene expression, yet when hubs assemble into larger, phase-separated condensates then gene expression is inhibited[87]. These results support a "Goldilocks" model whereby small changes in multivalent interactions can finely tune gene activation up or down, but where formation of condensates results in transcription inhibition[87]. Clearly, more work is required in this exciting area and examination of gene context will be critical, with condensates potentially enabling transcription at certain loci while restricting it at others. Additional technological innovations are therefore needed to further dissect the role of phase separation in transcription, including improved techniques to evaluate proteins by high resolution microscopy when expressed at endogenous levels in

live cells.

Regulation of RNA polymerase II via phase separation

RNA Pol II contains a conserved, intrinsically disordered C-terminal domain (CTD) that enables it to form condensates, as well as to co-phase separate with transcription factors and transcriptional machinery[88-91]. The longer length of the mammalian CTD relative to the *S. cerevisiae* CTD (52 v. 26 heptad repeats) results in an increased propensity to undergo phase separation due to stronger CTD-CTD interactions[88]. CTD phosphorylation leads to dissociation of Pol II from Mediator condensates (associated with transcription initiation) and recruitment into condensates containing splicing factors[91] (FIG. 5e). These transitions could involve direct maturation of Pol II-containing condensates or Pol II exiting initiation condensates before recruitment to splicing condensates[92].

Related to this work, Quintero-Cadena *et al.* showed that Pol II CTD length correlates with gene density in eukaryotes, and proposed that this domain serves as a molecular bridge for Pol II to be recruited to active promoters, with longer CTD lengths enabling recruitment across greater distances[93]. CTD length also modulated transcriptional bursting with longer CTDs leading to stronger and more frequent bursts. Surprisingly, a truncated *S. cerevisiae* CTD was non-functional but function was restored by fusion to phase-separating IDRs from human FUS or TAF15 proteins[93]. Together, these studies demonstrate that CTD-CTD interactions, as well as CTD interactions with TFs, coactivators, and Mediator, assist the recruitment of Pol II for gene transcription.

RNA plays an integral role in transcriptional phase separation; low RNA levels increase the formation of Mediator condensates whereas high RNA levels dissolve these condensates[94]. This may result in a feedback mechanism whereby transcription dissipates condensates and causes the transcriptional bursts that are characteristic of this process[94]. Moreover, promoter-associated RNAs in mammalian cells are likely bound by multiple RBPs that enable Pol II and cofactors to reach the threshold levels necessary for phase separation[95], and certain RBPs also promote Pol II release from

329 initiation complexes to support transcription elongation[96].

330 Recent studies in *S. cerevisiae* have similarly shown that TBP associated factor 14 (Taf14; a
331 component of TFIID and chromatin remodeling complexes) utilizes multiple interaction partners to
332 regulate transcription. Chen *et al.* demonstrated that a structured extra-terminal (ET) domain of Taf14
333 recruits co-factors via an ET-binding motif present on these partners[97]. Moreover, Taf14 formed
334 condensates *in vitro* and binding partners could partition into these droplets, establishing that Taf14
335 can act as a scaffold to bring together co-factors and drive gene expression[97]. While the formation of
336 phase-separated hubs is a recurring theme in transcription, Taf14 is, so far, an unusual example of a
337 transcriptional regulator that is reported to use only structured domains to scaffold multi-component,
338 phase-separated condensates[97].

339

340 ***Fungi as model species to study amyloid disease***

341 In addition to endogenous phase separation phenomena, fungi have been used to model the
342 properties (and toxicities) of aggregative amyloids implicated in human disease[98,99]. In many cases,
343 the precise role of condensate or amyloid formation in neurodegenerative diseases such as
344 Huntington's, amyotropic lateral sclerosis (ALS), Alzheimer's and Parkinson's is uncertain, although
345 disease-causing mutations often increase amyloid formation in proteins associated with each
346 disease[100,101].

347 Several aggregation-prone human proteins have been analyzed in *S. cerevisiae*, with high-
348 throughput screening used to identify new therapeutics that reduce amyloid toxicity[102,103]. Yeast have
349 also been invaluable is in studying disaggregases that can detoxify aggregation-prone proteins
350 associated with neurodegeneration. For example, *S. cerevisiae* Hsp104, or variants of this chaperone,
351 can not only act as disaggregases (or anti-aggregation activities) on endogenous proteins but are also
352 functional on human proteins associated with neurologic disorders despite metazoans lacking an
353 ortholog of Hsp104[104,105]. Model fungi and yeast genetics therefore continue to be used to understand

how aberrant phase transitions/aggregation can impact neurodegenerative diseases and to develop therapeutic interventions.

Conclusions

Phase separation and the formation of biomolecular condensates play central roles in virtually all aspects of biology, from ubiquitous cellular compartments to highly inducible assemblies. In fungi, the formation of condensates is highly sensitive to cellular conditions and phase separation therefore acts as an exquisite sensor of environmental cues, as evidenced in the responses to changes in pH, ROS and CO_2 levels. Outstanding questions with regards to phase separation and MLOs in fungi include a better understanding of the molecular interactions that nucleate and stabilize phase separation; the contribution of transient or stable secondary structures to condensate formation; understanding the specificity by which proteins/nucleic acids are recruited to condensates; new tools to examine phase separation of proteins at endogenous levels in fungal cells; determination of which small molecules impact condensates; and developing therapeutic approaches including antifungal drugs based on understanding of phase separation biology. Studies in fungi will continue to be at the forefront of this field given the cell biological and genetic tools available in model fungi and the diversity of species being studied both as model organisms and as plant and human pathogens.

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Contributions

MIS wrote the initial draft which was extensively revised by RJB and CF, with input from NLF.

Competing interests

NLF is a member of the scientific advisory board of Dewpoint Therapeutics.

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Super-enhancer features	Mammalian super-enhancers	*C. albicans* super-enhancer-like regions
Role in cell identity	Found to control cell identity and differentiation in murine embryonic stem cells (ESCs), multiple immune cell type, and to contribute to a broad range of cancers via enrichment at genes with oncogenic function.	Extended regulatory regions are required for white-opaque cell fate determination and additionally for control of biofilm formation, whereby cells transition between planktonic growth and communal growth.
Size	Median size is > 8 kb, whereas typical enhancers are ~700 bp.	For the white-opaque TRN, median size of upstream intergenic regions is > 7 kb, while average intergenic regions are ~ 557 bp.
TF enrichment levels	Elevated TF binding at constituent enhancers, increased cooperative transcriptional activation, and combined TF/coactivator enrichment ~10-fold higher than seen at typical enhancers.	Master TFs bind together at multiple positions across super-enhancer-like regions (see Fig. 5), although quantitative analysis of cofactor levels has not been performed.
Epigenetic marks	Relatively high levels of acetylation of histone H3 at lysine 27 (H3K27ac) are commonly used to define super-enhancers, sometimes in combination with other criteria.	Unknown.
Sensitivity to TF perturbation	Highly sensitive – blocking binding of just one coactivator, like BRD4, can collapse entire super-enhancer.	Highly sensitive – a small increase or decrease in levels of the Wor1 TF, for example, can drastically alter white-opaque cell fate switching rates.

Table 1. Comparison of super-enhancer features in mammalian cells with super-enhancer-like elements in *C. albicans* cells. White-opaque and biofilm transcriptional regulatory networks (TRNs) are used as examples of SE-like regulatory regions in *C. albicans*.

Figure Legends

Figure 1. Stress granule and P body formation in response to environmental changes.
Extracellular stress such as heat shock or a sudden drop in pH leads to global inhibition of translation and ribosome stalling. Stalled mRNAs are diverted to either P bodies or stress granules. Following pH stress, stress granules dissolve spontaneously, whereas stress granules formed following temperature stress require the assistance of Hsp40, Hsp70 and Hsp104. Subsets of mRNAs that are concentrated in P bodies can either be degraded or exchanged with stress granules.

Figure 2. The effect of molecular crowding on phase separation.
a, Microrheology using self-assembling Genetically Encoded Multimers (GEMs) allows measurement of intracellular crowding in the nucleus and the cytoplasm. Individual monomers consist of a *Pyrococcus furiosus* encapsulin scaffold fused to a fluorescent protein and spontaneously assemble into 40 nm spheres. The nucleus is a more crowded milieu than the cytoplasm and thus the random thermal motion of GEMs is decreased. **b,** mTORC activation following starvation results in an increased ribosome number. This increase in molecular crowding can increase phase separation of cytoplasmic proteins. Adapted from [30].

Figure 3. Phase separation in polarized growth, cell asymmetry and nuclear divisions.
a, Spa2 localizes Aip5 to the *S. cerevisiae* bud tip, recruiting Bni1 and nucleating actin filaments. Stresses such as low pH or energy depletion result in Aip5 and Spa5 forming cytoplasmic condensates. When both Aip5 and Spa2 are present, these condensates are rapidly disassembled following removal of the stress. In the absence of Spa2, Aip5 forms more stable condensates that are not readily disassembled and there is a consequent loss of viability following prolonged stress. Adapted from [34].
b, In *A. gossypii,* mRNA binding protein Whi3 forms distinct protein/mRNA condensates. Whi3

condensates formed with Bni1and Spa2 mRNA are localized to the site of branch formation (symmetry breaking) where they nucleate actin assembly. Whi3 droplets containing Cln3 mRNA form adjacent to the nucleus where they regulate asynchronous nuclear division (cell cycle regulation). Adapted from [36].

Figure 4. Phase separation regulation of bulk and selective autophagy.

a, When nutrients are abundant, Atg13 is hyperphosphorylated by TORC1 kinase. Upon nutrient starvation, Atg13 is dephosphorylated by PP2C phosphatase enabling interactions with the Atg17-29-31 complex and the Atg1 kinase which then coalesce into condensates. The resulting Pre-Autophagosomal Structure (PAS) is tethered to the vacuolar membrane by interactions with Vac8. In the newly formed PAS, Atg1 auto-phosphorylates itself and hyper-phosphorylates Atg13. Cytoplasmic phospho-Atg13 is trafficked back to the PAS following dephosphorylation by PP2C. Thus, Atg1 and PP2C maintain an equilibrium of unphosphorylated and phosphorylated Atg13 which maintains the PAS. The PAS is also the site of Isolation Membrane formation, which in turn will become the mature autophagosome. **Inset,** Following PAS formation, vacuoles containing ATG9 are recruited and subsequently fuse to become the cupped isolation membrane, which grows to engulf materials destined for destruction in the lysosome. **b**, Dodecamers of Ape1 undergo phase separation and are degraded by selective autophagy in nutrient rich conditions. The Ape1 condensate is coated by a shell of the Atg9 adapter protein which templates growth of Atg8-decorated isolation membrane. Ape1 mutants which form aggregates are not engulfed. Adapted from [47].

Figure 5. Phase separation regulates transcription and fungal cell fate.

a, Coordinated binding of multiple TFs to regions upstream of their ORFs is often observed even without consensus binding sites for these regulators, suggestive of recruitment by protein-protein interactions. **b**, A phase separation model of transcription where TFs form condensates together with the transcriptional machinery to regulate the expression of cell identity genes. **c**, *C. albicans* switches

epigenetically between “white” and “opaque” phenotypic states. **d**, The white-to-opaque transition is regulated by a TF network whose members bind to their own promoters as well as those of others in the network, as indicated by the arrows. Adapted from ref [82]. **e**, RNA polymerase II interacts with transcriptional initiation or elongation condensates depending on the phosphorylation state of its C-terminal domain (CTD).

Box 1. Molecular forces promoting phase separation

Multivalent interactions, in which a single component contacts multiple other components, are critical drivers of phase separation. Multivalency can be achieved by proteins with intrinsically disordered regions (IDRs) or can involve interactions between folded domains and short linear motifs (SLiMs) that are sequence-specific recognition sites[1,3,106-109] . Prion-like domains (PrLDs) are a particularly important class of IDRs and are defined by their sequence composition which is enriched in uncharged polar amino acids and glycine similar to *S. cerevisiae* prions.

A “sticker and spacer” model has been developed to describe how phase-separating IDRs interact. This simplified model defines “sticker" residues as those involved in electrostatic, hydrophobic, cation–Pi or Pi–Pi interactions, while “spacer” residues do not contribute to intermolecular interactions[109,110]. Attention has focused on aromatic residues as important “stickers” that can form Pi-Pi interactions with other aromatic residues or cation-Pi interactions with basic residues[107,109-111]. The nature of the spacer residues also defines biocondensate properties with glycine spacers promoting liquid behavior whereas glutamine/serine spacers promote solidifying of condensates[110].

Overall, many residue types and intermolecular forces can contribute to phase separation, such as hydrophobic contacts/amino acids that can drive condensation of IDRs[108,112,113]. Formation of transient structures (including β-sheets and intermolecular helical regions) can also increase self-

assembly and condensation formation[114-116]. In fact, one model proposes that transient structures are intermediate events in the formation of labile β-sheet structures that drive LLPS[116-118], although this model is contested[113,119].

Phase-separating molecules have further been designated as "scaffolds", that are both necessary and sufficient for phase separation, and "clients", which can selectively partition into condensates but do not phase separate by themselves[120,121]. Condensation also can be coupled to the formation of a system-spanning or "percolation" interaction network, although percolation networks can also occur independently of phase separation[122].

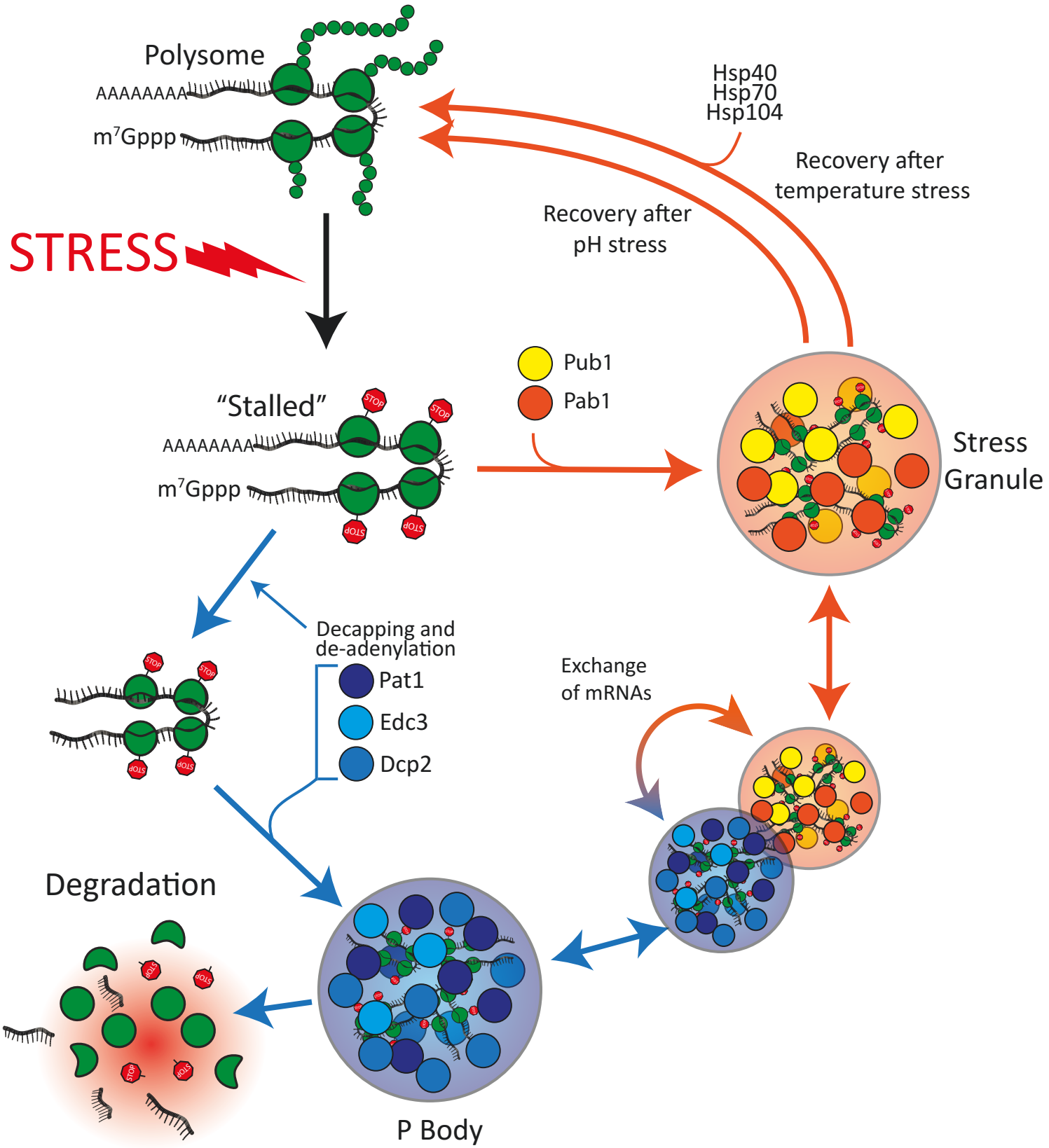
Box 1 Fig Legend. Weak protein-protein interactions and multivalency drive phase separation. **a**, Phase separation occurs when protein concentration, osmolarity, temperature, and/or pH cross a threshold where intermolecular interactions drive assembly into a dense phase that co-exists with the surrounding dilute phase (adapted from ref [123]). **b**, Higher valency due to a higher number of potential interactions between two peptide chains promotes phase separation. **c**, A "sticker" and "spacer" model for phase separation. Charged and aromatic "sticker" residues (larger balls) are distributed along a polypeptide interspersed with stretches of polar, hydrophilic residues (smaller balls) that act as "spacer" residues. **d**, "Scaffold" proteins have the capacity to undergo phase separation independent of other factors due to their high valency. Scaffolds can recruit "client" proteins that by themselves are not able to undergo phase separation under the same conditions.

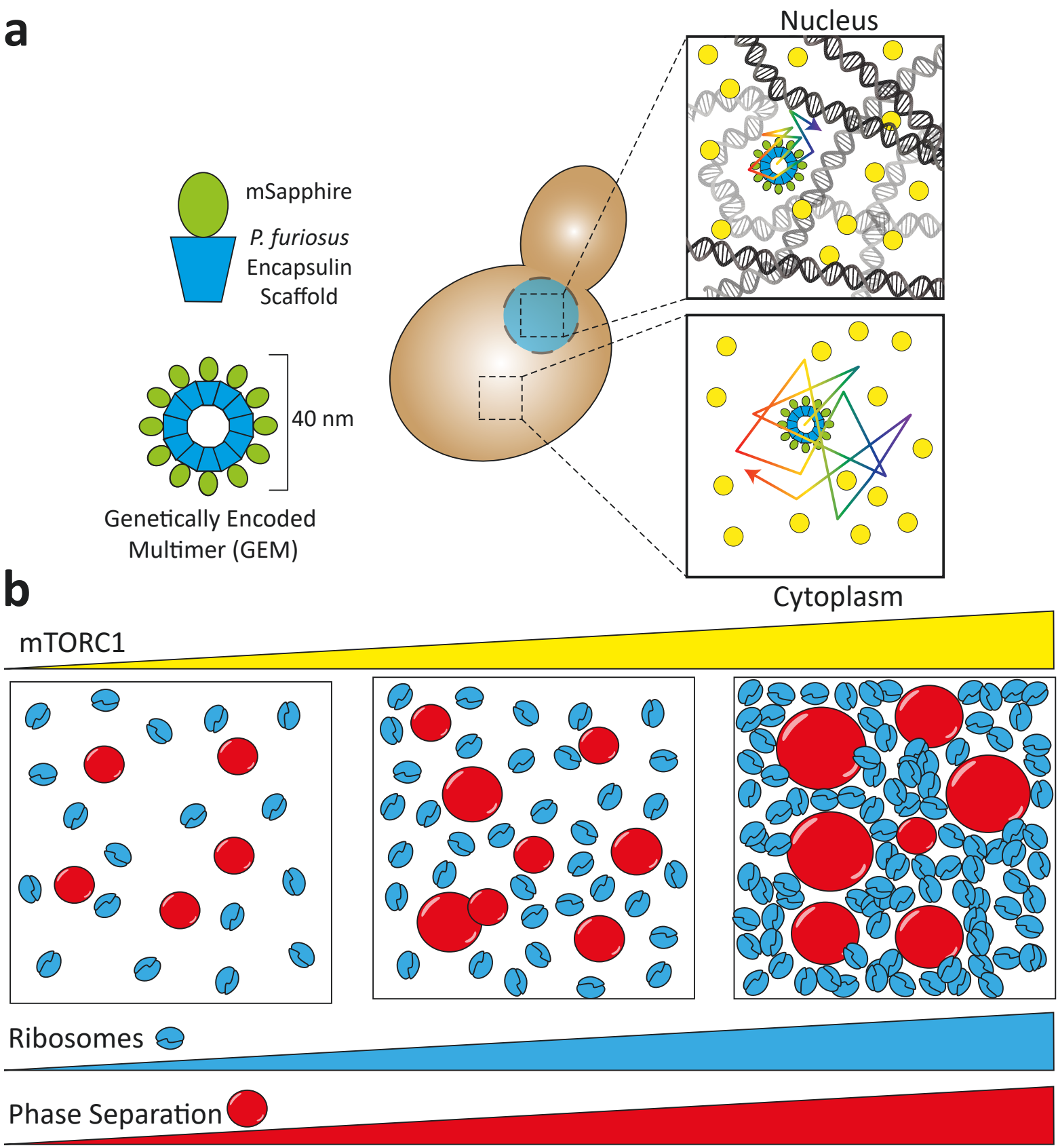
Box 2. Protein-nucleic acid interactions drive condensate formation

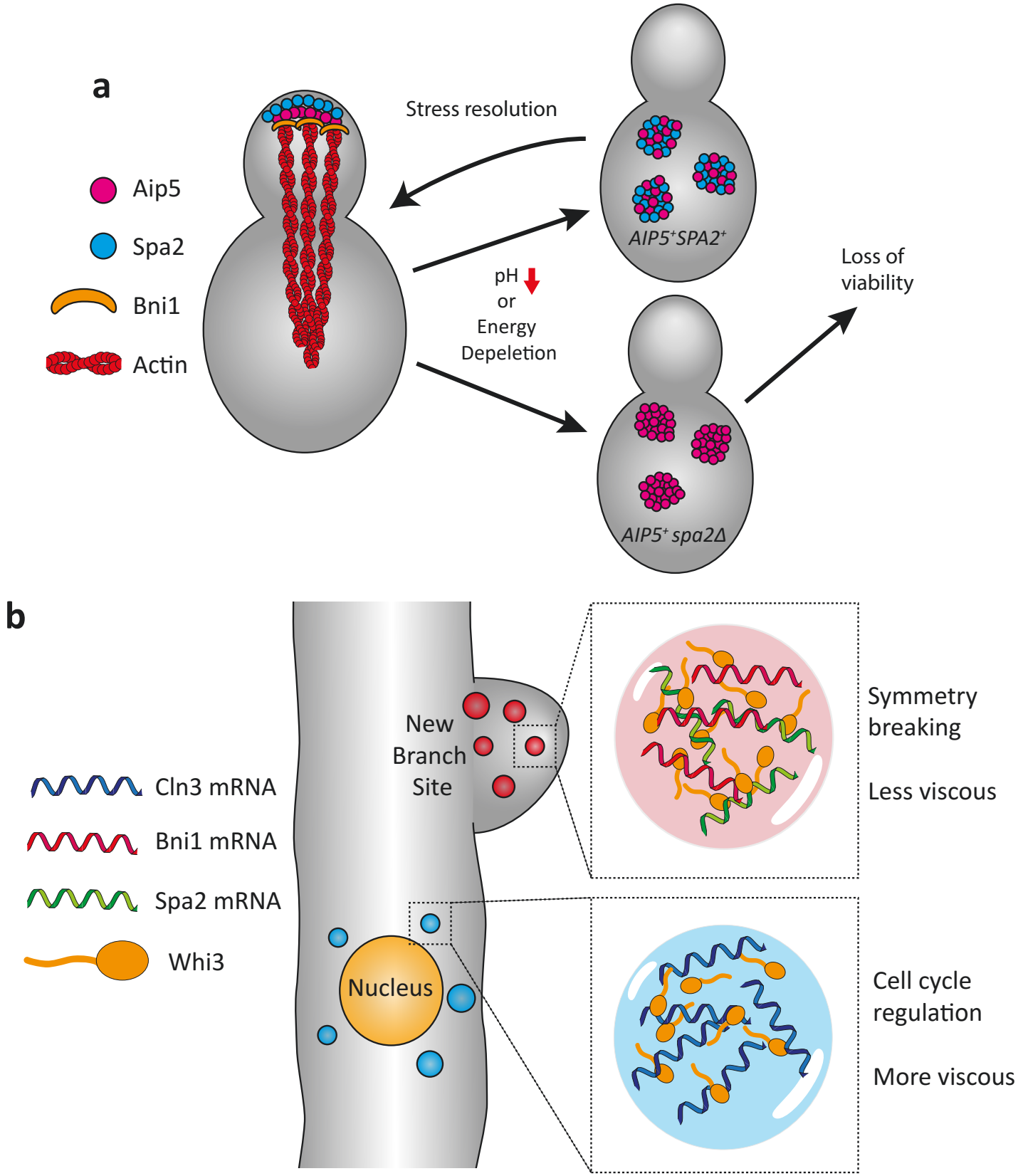
Negatively charged nucleic acids can interact with positively charged proteins to drive phase separation, a form of complex coacervation where multivalent nucleic acid-binding domains can support condensate formation even in the absence of IDRs[124]. Single-stranded nucleic acids can also participate in cation-pi or pi-pi interactions through exposed aromatic bases thereby supporting

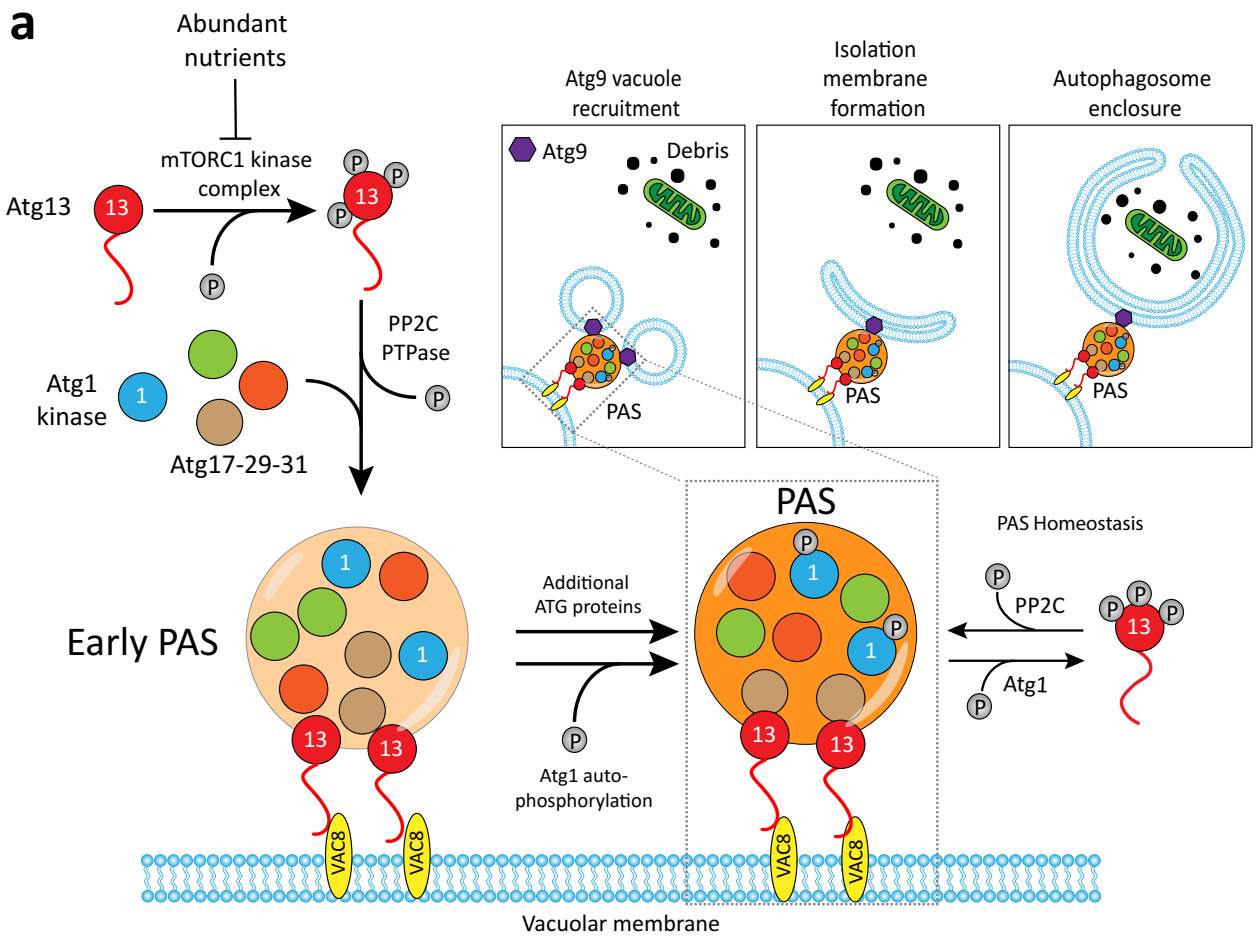
807 condensate formation, while recognition of nucleotide bases via hydrogen bonds is an additional
808 driving force for phase separation[125].

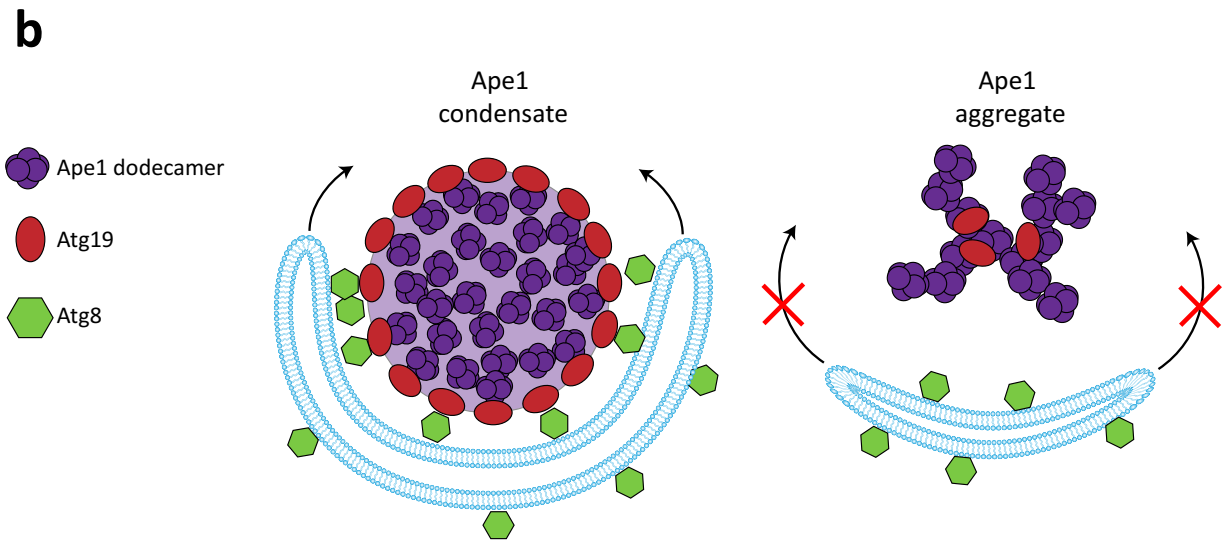
809 RNA is a critical component of multiple condensates including stress granules, storage
810 granules, and various speckles, paraspeckles, nuclear speckles and transcriptional complexes. RNA
811 transcripts can directly seed the nucleation of condensates or can induce protein conformations that
812 promote condensation[126,127]. RNA levels can also tune phase separation of ribonucleoprotein (RNP)
813 condensates. For example, low RNA levels stimulate condensate formation by human FUS protein
814 while high RNA levels suppress condensate formation, and changes in RNA concentrations may
815 underlie aberrant LLPS structures and pathologic assemblies[128]. While liquid condensates adopt a
816 spherical shape in the absence of external forces, certain RNP-containing condensates form highly
817 viscous structures, mesh-like assemblies or filamentous networks depending on the RNA substrates
818 involved, highlighting how various shapes and structures can arise via phase separation[39,129].
819

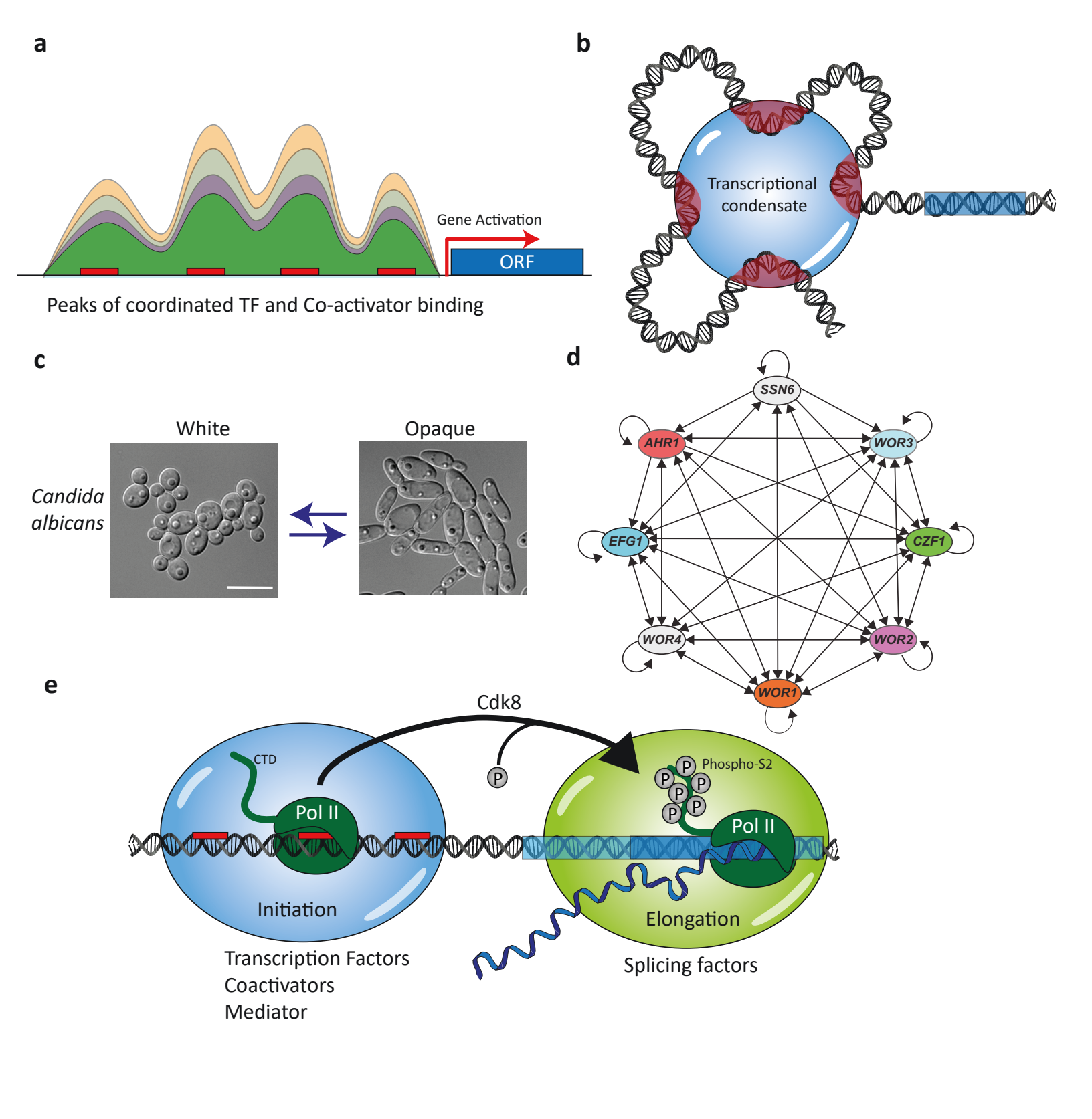

Polysome
AAAAAAAA
m7Gppp
STRESS
Hsp40
Hsp70
Hsp104
Recovery after
temperature stress
Recovery after
pH stress
"Stalled"
AAAAAAAA
m7Gppp
STOP
Pub1
Pab1
Stress
Granule
Decapping and
de-adenylation
Pat1
Edc3
Dcp2
Exchange
of mRNAs
Degradation
P Body

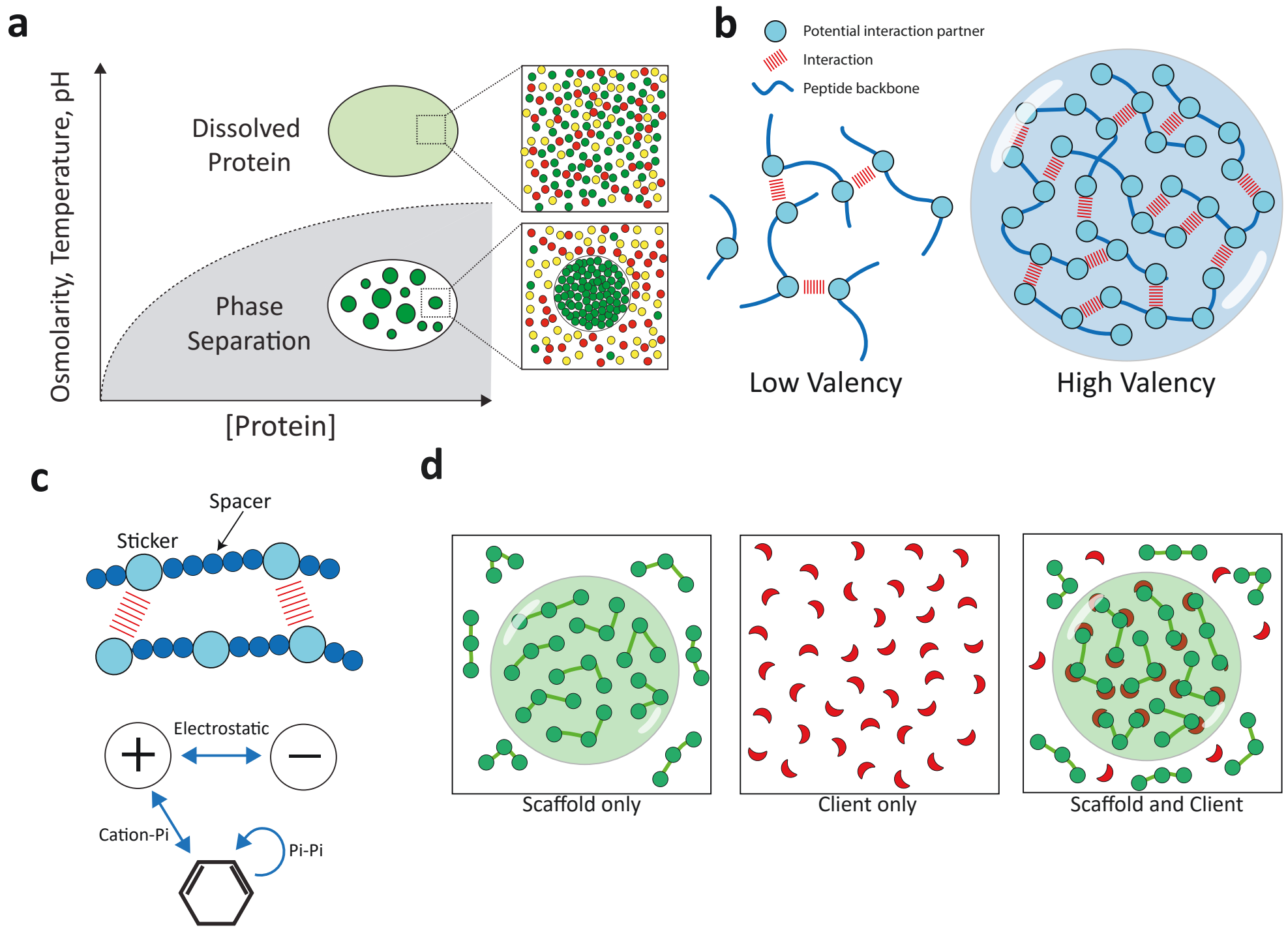

a
Nucleus
mSapphire
P. furiosus
Encapsulin
Scaffold
40 nm
Genetically Encoded
Multimer (GEM)
Cytoplasm
b
mTORC1
Ribosomes
Phase Separation


a
Aip5
Spa2
Bni1
Actin
Stress resolution
AIP5⁺SPA2⁺
pH
or
Energy
Depeletion
Loss of
viability
AIP5⁺ spa2Δ
b
Cln3 mRNA
Bni1 mRNA
Spa2 mRNA
Whi3
New
Branch
Site
Nucleus
Symmetry
breaking
Less viscous
Cell cycle
regulation
More viscous


a
Abundant nutrients
mTORC1 kinase complex
Atg13
13
P
Atg1 kinase
1
Atg17-29-31
PP2C PTPase
Atg9 vacuole recruitment
Isolation membrane formation
Autophagosome enclosure
Atg9
Debris
PAS
Early PAS
Additional ATG proteins
Atg1 auto-phosphorylation
PAS Homeostasis
PP2C
Atg1
VAC8
Vacuolar membrane


b
Ape1 dodecamer
Atg19
Atg8
Ape1 condensate
Ape1 aggregate


a
Gene Activation
ORF
Peaks of coordinated TF and Co-activator binding
b
Transcriptional condensate
c
White
Opaque
Candida albicans
d
SSN6
AHR1
WOR3
EFG1
CZF1
WOR4
WOR2
WOR1
e
Cdk8
CTD
Pol II
P
Phospho-S2
Initiation
Elongation
Transcription Factors
Coactivators
Mediator
Splicing factors


a
Osmolarity, Temperature, pH
Dissolved Protein
Phase Separation
[Protein]
b
Potential interaction partner
Interaction
Peptide backbone
Low Valency
High Valency
c
Spacer
Sticker
Electrostatic
Cation-Pi
Pi-Pi
d
Scaffold only
Client only
Scaffold and Client