Review Article

Biochemical Timekeeping Via Reentrant Phase Transitions

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Abstract

Appreciation for the role of liquid–liquid phase separation in the functional organization of cellular matter has exploded in recent years. More recently there has been a growing effort to understand the principles of heterotypic phase separation, the demixing of multiple proteins and nucleic acids into a single functional condensate. A phase transition is termed reentrant if it involves the transformation of a system from one state into a macroscopically similar or identical state via at least two phase transitions elicited by variation of a single parameter. Reentrant liquid–liquid phase separation can occur when the condensation of one species is tuned by another. Reentrant phase transitions have been modeled *in vitro* using protein and RNA mixtures. These biochemical studies reveal two features of reentrant phase separation that are likely important to functional cellular condensates: (1) the ability to generate condensates with layered functional topologies, and (2) the ability to generate condensates whose composition and duration are self-limiting to enable a form of biochemical timekeeping. We relate these biochemical studies to potential cellular examples and discuss how layered topologies and self-regulation may impact key biological processes.

Reentrant Phase Transitions of Protein and RNA

Biomolecular condensates form by phase separation to functionally organize proteins and nucleic acids without an encompassing membrane.[1,2] Aberrant phase transitions underlie disease states in conditions as diverse as neurodegeneration,[3–6] viral infection,[7–10] developmental disorders,[11,12] and cancer.[13–15] Conversely, functional and adaptive roles for condensates are also being rapidly identified across kingdoms and include RNA localization,[16–18] signal transduction,[19,20] genome organization,[21–23] gene transcription,[24–27] environmental sensing,[28,29] cell fate decision making,[30,31] chaperoning complex assembly[29,32] and accelerating biochemical reactions.[33] In physics, a system experiences reentrant phase transitions when variation of a single thermodynamic parameter results in two (or more) phase transitions that enable attainment of a state, which is macroscopically similar or identical to the original state in terms of the number of different phases and their respective material properties.[34,35] (Figure 1). In biology, some biomolecular condensates form and dissolve via a reentrant liquid–liquid phase separation (LLPS) whereby the condensation of one molecule is tuned by the relative concentration of another[36] (Figure 1). An archetypical example is the phase separation of an RNA-binding protein (RBP) tuned by a cognate RNA (Figure 1). Here, the system returns to a macroscopically similar state, consisting of a single light phase, that differs on the molecular level from the initial state in terms

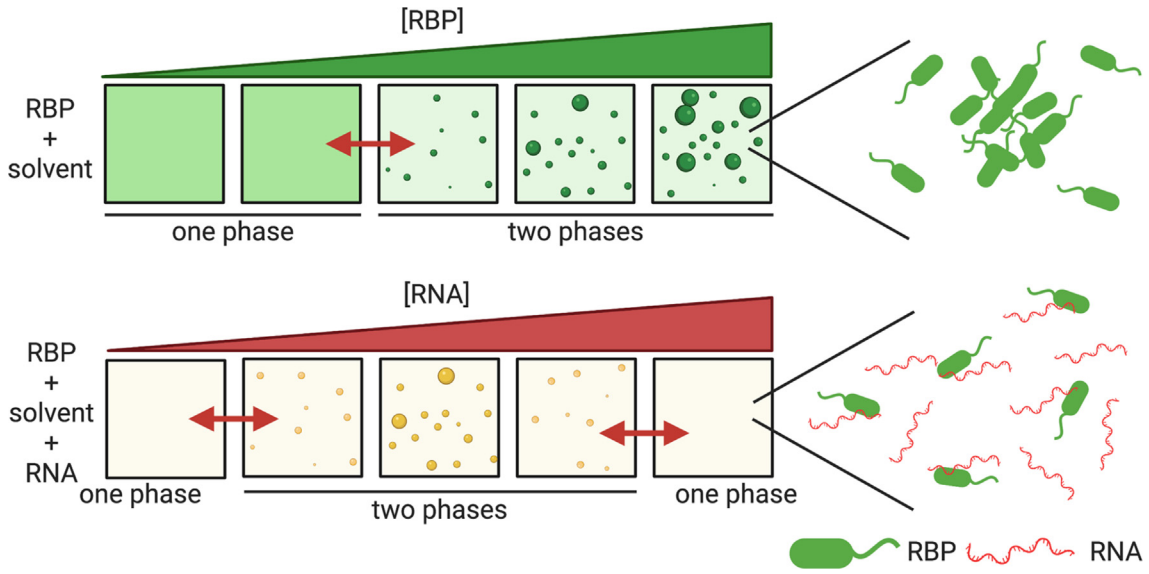

Figure 1. Reentrant phase separation of a heterotypic condensate. For a homotypic condensate (top panel) composed a single RNA binding protein (RBP), a phase transition occurs (red arrow) when [RBP] > C_{sat}, leading to a transition from a one-phase to a two-phase solution consisting of a light phase depleted of the RBP and a RBP rich dense phase. Increasing the RBP concentrations leads to an increase in the volume fraction of the dense phase, depicted by increased condensate size and number as a function of RBP concentration. An example of a heterotypic condensate (bottom panel) composed of the same RBP and a co-condensing RNA. Here, [RBP] < C_{sat} and is fixed. Increasing the concentration of RNA drives an initial phase transition (left red arrow) and the formation of heterotypic condensates composed of both the RBP and RNA. As RNA concentration increases, condensates dissolve via a second, reentrant phase transition (right red arrow), leading to a one-phase solution macroscopically similar to the first. At the molecular level, this solution consists of RBP + RNA complexes, and unbound RNA.

of its components and their interactions (Figure 1). Reentrant phase behavior is thus ideally suited to engender condensates that regulate the duration of their own existence and time the biochemical processes they facilitate.

Homotypic LLPS results in the switchlike demixing of a biomolecule into a high-concentration dense phase and low-concentration light phase.[37] Demixing occurs when the saturation concentration, C_{sat}, of the molecule is reached[37] (Figure 1). This process is thought to underlie the formation of a growing list of membraneless organelles (MLOs) in cells both in the nucleus and cytoplasm.[1,38] In cells, however, MLOs are highly heterotypic, and are composed of a complex mixture of both proteins and RNA.[39,40] Heterotypic condensates containing RBPs and RNA can exhibit reentrant phase behavior[36,41] (Figure 1). In such systems RNA can drive the phase transition of a RBP at concentrations where the RBP alone would not condense (Figure 1). The further addition of RNA leads to a second phase transition whereby condensates dissolve resulting in solution consisting of only the 'light phase'. Here, the initial and final solutions are macroscopically similar, consisting of only one phase with similar material properties, but differ on the molecular level (Figure 1). Reentrant LLPS can manifest as condensates with spatial organization in the form of layered topologies.[42,43] Additionally, reentrant LLPS could enable temporal regulation in the form of metastable condensates whose existence is self-limiting,[36] governed by the synthesis or decay of a second molecule that tunes the phase behavior of the first (Figure 2).

Reentrant behavior will emerge for multicomponent systems where homotypic and heterotypic interactions are in competition and are thus not limited to protein and RNA mixtures.[44–47] Examples of reentrant LLPS have been reconstituted biochemically, with compelling implications for cellular condensates. One such example is the prion-like domain (PrLD) containing RBP, FUS.[48–52] Chromosomal translocations can result in the PrLD of FUS being fused to DNA-binding domains, creating an oncogenic fusion protein that drives pathologic gene expression programs in sarcoma.[53,54] The PrLD of FUS can interact with the disordered C-terminal domain (CTD) of RNA polymerase II (Pol II) to activate transcription.[55–58] FUS, and its isolated PrLD, are archetypal examples of protein LLPS both *in vitro* and in cells.[3,58,59] FUS LLPS is also an example of reentrant LLPS modulated by RNA.[55,58,60] RNA lowers the C_{sat} of FUS, driving phase separation at lower FUS concentrations.[55,58,60] However, increasing amounts of RNA relative to FUS also serve to inhibit FUS condensation.[58] Notably, RNA also regulates the association

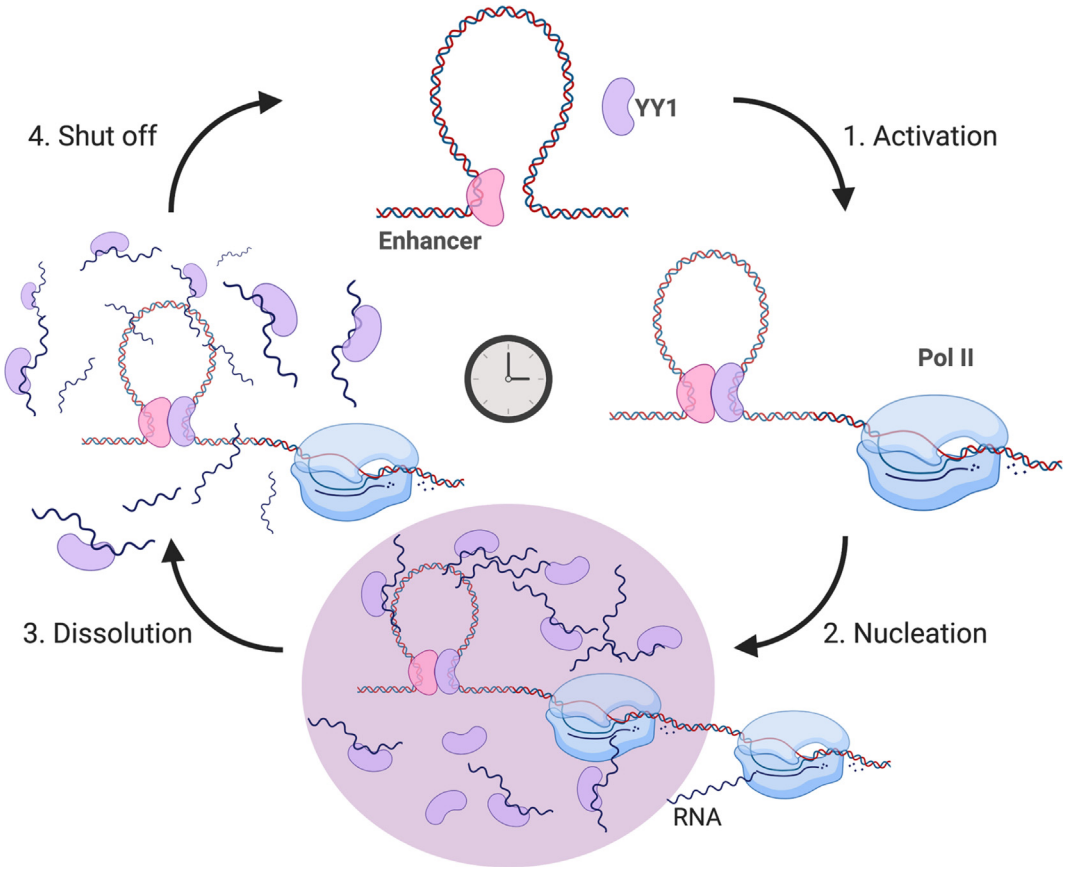

Figure 2. A model for self-limited transcription via reentrant LLPS. 1. An initial enhancer-promoter contact is formed involving the factor YY1, leading to Pol II recruitment and gene activation (right). 2. As more RNA is produced, it entraps YY1 and the high local concentration nucleates a transcriptional condensate rich in YY1 (bottom center). 3. Increasing mRNA production leads to a reentrant phase transition resulting in dissolution of the transcriptional condensate (left). 4. The reduced local YY1 concentration leads to a loss of DNA binding, and the gene is shut off (top). Thus, phase separation peaks after nucleation and wanes as the RNA concentration increases providing temporal regulation of transcription (indicated as a clock).

between FUS and the Pol II CTD.[55,58] In this sense, FUS:CTD interactions could initially elicit the production of mRNA, further stabilizing the FUS:CTD interaction via the formation of FUS condensates that recruit Pol II, driving more transcription via a feed-forward mechanism. Conversely, as RNA is transcribed and accumulates locally, the FUS condensate could be dissolved, creating a feedback mechanism.

Specific RNAs could also play roles in regulating reentrant phase behavior in cells. This phenomenon has been observed for the PrLD containing RBP, Whi3, from the multinucleated filamentous fungus, *Ashbya gossypii*.[16,61] *In vitro*, Whi3 condensation is stimulated by the addition of a specific Whi3-bound transcript, *CLN3* but not by the addition of preparations of total RNA. Reentrant LLPS behavior is observed for Whi3 as a function of both *CLN3* and *BNI1* transcript concentration. Thus, Whi3 droplets appear, grow in size, and then shrink as a function of increasing amounts of each transcript. Notably, the physical properties of Whi3 condensates, including how readily they fuse, differ depending on whether *CLN3* of *BNI1* is added,[16] which has implications for the subcellular localization of these transcripts *in vivo*.[16,17] Both *CLN3* and *BNI1* harbor the same number of Whi3-binding sites with differences in the resulting condensate behavior owing to differences in the structure of each RNA, not the effective valency of the Whi3:RNA complexes.[17] This observation suggests an additional layer of complexity may regulate the reentrant phase behavior of RBPs in cells, with distinct RNAs modulating condensates with distinct properties and functions. One possibility is that the three-dimensional folds of specific RNAs act as structural scaffolds to bind and present RBPs in a specific spatial orientation relative to one another. Proximal arrangement of bound RBPs could favor interactions with one another in the same complex while more distal orientations could favor interactions with RBPs in the surrounding solution. Such distinct complexes could nucleate condensates with different compositions or material

properties. Likewise, short RNAs lacking complex three-dimensional structure and high valency could tune RBP phase separation by acting to limit valency or reduce the effective concentration of the RBP available to multimerize on longer transcripts. This concept has been explored with another PrLD containing RBP, TDP-43 that exhibits reduced aggregation in cells treated with short 'bait RNAs' targeting TDP-43.[5]

Multi-Layer Condensates

Reentrant LLPS can manifest as condensates with layered topologies. This phenomenon has been modeled *in vitro* using RNA:protein and RNA:peptide mixtures that undergo a charge-mediated phase transition known as complex coacervation.[41–43,62] In these studies, layered condensates emerge as a function of RNA:protein ratios, and the sequence composition of the components.[41–43,62] A leading cause of the neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) is a hexanucleotide repeat expansion in the *C9orf72* gene that can lead to the expression of arginine-containing dipeptide repeats (DPRs).[63] Proline-arginine (PR) DPRs form spherical liquid coacervates in the presence of poly-A, poly-U and poly-C RNAs.[62] However, the addition of poly-G RNA which is capable of forming G-quadruplex structures, induces the formation of mesh like aggregates.[62] Additionally, mixing poly-A and poly-C RNA, and altering the ratio of this mixture relative to PR DPRs, yielded layered condensates with a core–shell architecture when poly-A:poly-C ratios were close to 1.[62] Conversely, well mixed PR:RNA condensates formed when either RNA species was in excess over the other.[62] In addition to core–shell topology, protein-RNA reentrant LLPS can also create hollow condensates in certain protein:RNA regimes. This phenomenon has also been demonstrated for the ALS/FTD-linked RBP, FUS in the presence of poly-U RNA[42] which forms hollow condensates reminiscent of the vacuolated droplets formed by ALS/FTD-linked protein, TDP-43 when it is overexpressed in cells.[64] As these studies show, even simple two and three component reentrant systems can encode the formation of multi-layer condensates whose architecture responds to the concentration of each component. The extent to which such systems faithfully model complex cellular condensates composed of longer RNAs of more complex sequence, is an exciting area of future research. What is already apparent is that condensates exist in cells with features reminiscent of reentrant systems. One such condensate is the nucleolus, whose layered topology is intimately related to function.[65–67]

Nucleoli are bioreactors for ribosomal RNA (rRNA) transcription and ribosomal subunit assembly. They are also liquids, demixed from the surrounding nucleoplasm and maintained without an encompassing membrane.[65,68] Moreover, nucleoli are themselves a series of nested and immiscible liquids consisting of three layers.[66] The innermost layer is the fibrillar center (FC) where rRNA transcription occurs.[66] The FC is surrounded by the dense fibrillar center (DFC) rich in the protein fibrillarin and the site of rRNA processing.[66] The FC and DFC are nested with the granular component (GC) where ribosomes are assembled, which is enriched with the protein nucleophosmin.[66] Nucleophosmin and fibrillarin condensates can be reconstituted *in vitro* in the presence of rRNA.[66,69] Disrupting ATP-dependent processes, including transcription, in nucleoli *in vivo* alters their biophysical properties.[66,68] Further, blocking transcription with the drug actinomycin D causes nucleoli to shrink, as would be expected of a reentrant phase transition modulated by RNA.[67] The layered architecture of the nucleolus amounts to an assembly line that directs the assembly of mature ribosomal subunits and their flux out of the nucleolus.[67] Remarkably, the layered environment of the nucleolus may serve as an elaborate *chaperone* to ensure the correct assembly of rRNA into ribosomes.[67] Nascent rRNA is initially unfolded and accessible to protein binding by nucleolar RBPs and thus retention in a given nucleolar layer.[67] rRNA processing and folding gradually reduces the effective valency of rRNA for nucleolar RBPs and reduces retention in a given nucleolar layer, enabling partitioning into the next.[67] In this sense, the layered nucleolus may *time* the processing of rRNA and prevent premature flux out of the nucleolus before correct rRNA folding is achieved.[67]

Biochemical Timekeeping in Transcription Regulation

Might other examples of molecular timekeeping through reentrant phase separation occur in cells that tie the duration of a biochemical process to the existence or composition of a condensate? Protein-coding gene transcription can occur in condensates composed of transcription factors and coactivators[25,27,70,71] and the clustering of Pol II via its disordered CTD.[24,72–74] The dynamics of such clusters are predictive of the output of transcription.[71,75] If one or more scaffolding components of a transcriptional condensate undergo reentrant phase separation tuned by RNA, bursts of active transcription at a given gene could be self-limiting.

This possibility has yet to be directly observed in live cells, yet compelling evidence exists that suggests self-limiting transcriptional activity may occur. For example, Yin Yang 1 (YY1) is a transcription factor (TF) with a zinc-finger DNA-binding domain that interacts with CTCF and serves to link enhancers to gene promoters to regulate transcription.[76] YY1 interacts with

enhancers, which are proposed to be liquid compartments[25] and has an intrinsically disordered region (IDR). IDRs can facilitate LLPS when they harbor multivalent interaction motifs or 'stickers'.[1,77–79] Notably, YY1 binds RNA in addition to DNA, and RNA binding promotes its localization to enhancers and active gene promoters with RNA facilitating enhanced DNA occupancy.[80] YY1 entrapment by nascent RNA could provide a local pool of YY1 at an active promoter, which could exchange with DNA in a feedback-loop to maintain transcriptional activity.

But what about gene shut off? A reentrant phase transition by YY1 regulated by nascent RNA from enhancers or promoters could provide a means to create the local pool of YY1 for DNA binding and continued activation, as well as enable shut-off when a threshold level of mRNA is produced **(**Figure 2**)**. In this model, enhancer associated YY1 comes into proximity with a promoter, contributing to an enhancer-promoter loop and gene activation.[76] Nascent mRNA from the activated promoter recruits more YY1, nucleating a promoter-associated condensate, as has been observed for other transcription factors **(**Figure 2**)**.[25,27,81] As more RNA accumulates locally from the active gene, YY1:RNA interactions would become increasingly favored, ultimately leading to the dissolution of the YY1 condensate and down-regulation of the associated promoter **(**Figure 2**)**. YY1 binds active promoters broadly, and other DNA-binding TFs have also been shown to interact with RNA,[80] suggesting that a self-limiting model for transcription activation through reentrant phase transitions could be a widespread phenomenon. Additional regulation of the reentrant phase could be encoded by the relative strength of TF:TF, TF:DNA, TF:RNA interactions and the number of TF-binding motifs present in a given promoter or associated nascent mRNA.[82] Likewise, pathologic transcription factors, such as those created by FUS PrLD fusion to DNA-binding domains, could escape such controls. Finally, altered ratios of components as a function of mRNA production and factor recruitment could not only dissolve the phase, but alter its composition as a function of time. This process would enable a transcriptional activation condensate to 'mature' into one harboring elongation and splicing machinery and perhaps ultimately termination machinery.[24]

Gene transcription may provide a more complex example of reentrant behavior where condensate nucleation and dissolution could be governed by distinct polymers, poly-ADP ribose (PAR) and RNA. PAR has been implicated in transcription activation[83,84] and PAR chains mimic RNA and DNA to engage transcription factors.[84,85] Highly activated promoters of heat-shock genes become enmeshed in PAR chains which serve to recruit transcription machinery to the gene locus at levels in excess of those required to saturate the underlying DNA.[84] This finding led to the hypothesis that PAR cages create a 'transcription compartment' to drive robust heat shock gene expression.[84] This compartment could serve the dual role of shielding the heat-shock loci from the broader transcriptionally repressed environment of the nucleus during times of stress.[84,85] For cellular function to return to normal, stress-induced transcriptional programs must eventually give way to homeostatic ones. How might PAR-caged heat shock genes be shut off? One mechanism could involve degradation by PAR glycohydrolases.[84] Alternatively, the heat-shock transcription compartment itself may represent a reentrant condensate, nucleated by PAR but dissolved by RNA. This model is compelling as recent studies implicate PAR in nucleating FUS condensates at sites of DNA damage,[86] and regulating cellular condensates more broadly.[87–89] The extent to which sites of activated transcription represent liquid condensates nucleated by PAR, and the manner in which these condensates are regulated to tune gene output, are interesting areas of future research.

Unlike single-component LLPS, reentrant LLPS enables multiple additional layers of regulation and functionality. One feature is structure, with hollow or layered topologies capable of emerging from systems with few components. One example may be the multi-layered nucleolus, whose structure governs the biogenesis, processing and exportation of ribosomal subunits. Reentrant phases can also enable biochemical processes reliant on the phase to be timed and self-regulating. Biological timekeeping of transcriptional burst duration could be one process governed by reentrant behavior, particularly if TFs moonlight as RBPs. As the field seeks to identify functions associated with condensates, answering questions about how functional structures and cellular timekeeping mechanisms may emerge from reentrant phases is an exciting avenue for exploration.

While this paper was under review, Young and colleagues provided experimental evidence in support of the concept of RNA-mediated negative feedback mechanism in transcription.[90]

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