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REVIEW

Critical transitions and tipping points in EMT

Peng Wang[1,4], Luonan Chen[2,3,4,5,*]

[1] Key Laboratory of Computational Biology, CAS-MPG Partner Institute for Computational Biology, Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[2] Key Laboratory of Systems Biology, Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai 200031, China
[3] Center for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, Kunming 650223, China
[4] School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China
[5] Institute of Brain-Intelligence Technology, Zhangjiang Laboratory, Shanghai 201210, China
* Correspondence: lnchen@sibs.ac.cn

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***Background*: Phase transition and phase separation as well as their tipping points are penetrating phenomena in biology and are intrinsic properties of biological systems ranging from basic molecule complexes to cells and all way up to entire ecosystems.**

***Results*: For example, phase separation has been established as a key mechanism for biological molecules such as protein or RNA to form membraneless organelles to perform complex biological functions. Phase transitions are commonly observed during cellular differentiation, and generally, there are the tipping points or critical states just before the phase transitions. And the stability of ecosystem and extinction of species are systematic manifestation of phase transitions. All phase transition and phase separation phenomena display switch-like behavior and critical transitions.**

***Conclusion*: Here we summarize the concepts regarding the epithelial-to-mesenchymal transition (EMT) as a type of phase changes and the implication of critical transitions in EMT, and discuss open questions and challenges in this fast-moving field.**

Keywords: EMT; phase transition; tipping points

Author summary: Similar to physical matters, such as water, living things are also dynamic and often transit through different states. For example, people can exit healthy state and develop diseases. The dynamics of life is driven by changes of macromolecules and the networks through which macromolecules interact. Recently, phase separation, a specific form of macromolecule state changes, emerges as a fundamental way to modulate the function of macromolecules. Intriguingly, phase separation shares similar characteristics with the state transition of biological networks. Here we compare molecule and network level state changes, and discuss the emerging principles of biological state transitions.

OVERVIEW OF PHASE-TRANSITION AND PHASE SEPARATION IN BIOLOGICAL SYSTEMS

The term phase transition is first used to describe state changes of physical systems, such as the transition of H_2O from solid to liquid and to gas phase when temperature or pressure raises [1]. During phase transition, matters at each state share uniform properties, which are typically modified as matters transit into a different phase. The continuous variation of temperature also allows the co-existence of different states, a phenomena termed phase coexistence, resulting in matters with mixed properties [1]. A hallmark of phase transition is the existence of critical states or tipping points [2,3], also called catastrophic bifurcations, around which the physical properties of the system change dramatically when external conditions breach specific thresholds [1,4].

Beyond physics, complex systems from a wide range of

fields can be studied from the perspective of phase changes and critical transitions. In economics, critical transition happens during a stock market collapse or a bank run. In ecology, the extinction of species or the collapse of an ecosystem can be studied with critical transitions, where a sudden catastrophic change from one stable state to another stable state occurs [5,6]. In these complex systems the degree of homogeneity (for example how many species in an ecosystem) and connectivity (how these species interact) are key parameters to determine the presence of critical transitions [7]. In systems that are modular, hence lack of connectivity and disconnected regions dominate, there is no critical transition, the system will gradually collapse characterized by independent collapses of smaller connected elements. On the other hand, systems that are highly connected demonstrate strong robustness against small perturbations and are able to "repair" local perturbations, resulting in global stability. However, the connected systems do possess critical transitions where strong perturbations beyond repair are propagated through the system, resulting in critical slowdown and cause a critical transition [6,8].

A special form of phase transition, phase separation is especially relevant in biological systems. Phase separation is the conversion of a single-phase system into a multiphase system, especially the separation of a single-liquid-phase solution into two coexisting immiscible liquids. Biological functions are carried out by macromolecules such as DNA, RNA and proteins, which possess tremendous sequence, structure and functional diversities. To carry out highly specific functions utilizing these diverse molecules, cells have evolved to form membraned compartments within cells, known as organelles, in which specific molecules are organized to perform specific functions. These organelles are crucial for the normal functionality of cells. For example, lysosomes are spherical vesicles with hydrolytic enzymes to break down biological molecules, and their functions are essential for cell's metabolism and defense against pathogens. The endoplasmic reticulum (ER) is another well-known organelle that is essential for protein biogenesis. Beyond membrane organelles, the interior of cell is traditionally considered to be a solution of molecule mixtures where key biological reactions occur through diffusion and random interactions. Recently, this naïve picture changed dramatically due to a large number of studies that demonstrated the existence of membraneless organells. These paradigm shifting observations demonstrated that the interior of the cell is not a random mixture of biological polymers, instead macromolecules form organized structure through intracellular phase separation, much like the formation of liquid droplets inside an aqueous environment. The biological, physical and chemical properties inside and outside of the membraneless organelles differ dramatically, allow the membraneless organelles to carry out specific biological functions and prevent other biological process to occur. Although there is no membrane, the interface of the two phases forms a barrier, which is analogous to the critical points during phase transition, across which the properties of the molecules change dramatically.

PHASE SEPARATION OF BIOLOGICAL MOLECULES

Currently there are a large number of membraneless organelles that have been documented. The majority of known membraneless organelles reside in nucleus including nucleolus, paraspeckles, snurposomes, PML bodies etc. The largest and best studied membraneless organelle is the nucleolus. The nucleolus is a complex compartment in the nucleus of eukaryotic cells which consists of three distinct structures: the fibrillar centers (FC), dense fibrillar component (DFC) and granular component (GC). The nucleolus is formed by proteins and RNAs around specific region of chromosome and plays key roles in ribosome biogenesis. Moreover, the structure of nucleolus changes dynamically in response to signal and during mitosis, suggesting that nucleolus may play important roles in cell cycle and cell's response to stress. Another well-established membraneless organelles in nucleus is paraspeckles, compartments located in the interchromatin space that play important roles in regulating gene expression and splicing. Another membraneless organelles closely related to paraspeckles is snurposomes, also known as nuclear speckles. The composition of snurposomes is highly enriched in pre-mRNA splicing factors, such as small nuclear ribonucleoproteins (snRNPs) and serine/arginine-rich (SR) proteins. Moreover, snurposomes are located near sites of active transcription, suggest that snurposomes play essential role in regulating alternative splicing. Several different types of membraneless organelles were also found in the cytoplasm such as P-bodies and stress granules, these condensates are typically made of RNA and protein complexes and all play important roles in regulating mRNA metabolism and homeostasis.

Interestingly membraneless organelles form a dynamic interacting network. Different types membraneless organells such as paraspeckles and snurposomes, or numerous mRNP granules in cytoplasm, share protein and mRNA components. Moreover, organelles also often share similar physical locates in the cells, and experimental evidences suggested that they could physically interact and exchange components, and form a regulatory cascade to perform key biological functions. A paradigm of systematic interactions of membraneless organelles is the

dynamic interplay between P-bodies and stress granules. P-bodies are uniqutious membraneless organelles that play critical roles in mRNA transport, modification and transportation. The macromolecules enriched in P-bodies include proteins involved in mRNA metabolism, and critically, RNA components that serve as scaffold to assembly the biological polymer complex. Stress granules are membraneless organless that also play essential functions in mRNA and protein metabolism. As the name suggests, stress granules are induced in response to stress signals and play essential roles in modulating RNA and protein metabolism. Importantly, P-bodies and stress granules demonstarte extensive compositional overlap, and their formation are closely linked. For example, studies in yeast demonstrated that yeast strains with deficiency in P-bodies were unable to induce stress granules, suggesting that P-bodies are prerequisite of functional stress granules, highlighting an intrinsic interplay between different membraneless organelles.

BIOLOGICAL FUNCTIONS OF PHASE SEPARATION OF BIOLOGICAL MOLECULES

The biological function of membraneless organelles is an area under active investigation. Currently, several emerging principles have been established. A prominent characteristic of molecules in phase separation compartment is their unusual concentration. For example, Li *et al*. (2012) showed that the concentration of proteins inside the liquid drops is approximately 100-fold higher than in the surrounding aqueous medium. The high concentration of macromolecules in membraneless organelles may promote their biological functions. A natural consequence of high concentration of macromolecules is the formation of multimeric congregates, and cells seem to use this principle for the formation of cellular structures. For example, increased concentration above threshold can cause tubulin to aggregate which leads to the formation of microtubule, which is an essential part of cytoskeleton that play essential roles in key biological processes such as cellular transportation, the movement of secretory vesicles, and mitosis. In a similar fashion, nucleation of actin molecules leads to the formation of microfilaments, the flexible framework that play essential roles in cell motility. Beyond cellular structures, dynamic phase separation of signal receptors also plays essential roles in signal transduction. The recognition of pathogenic antigens in the form of a short peptide epitope-MHC complex by T cell receptor (TCR) is the first, and essential step of immune response. Although millions of MHC-epitope complexes are typically present on the surface of an antigen presenting cell, the epitope corresponding to a specific pathogen, is very limited and could be numbered only in single digits. Hence TCR need to possess remarkable sensitivity towards these antigens to enable proper immune response. Phase separation plays an essential role in this fundamental process. It has been well established that TCR forms tight clusters upon contact with APC. Immunofluence staining experiments demonstrated that virtually all TCR of an APC-contacting T cell are focused on a single cluster on the site interacting with APC. This cluster of TCR demonstrated antigen scanning sensitivity and efficiency orders of magnitude higher than unclustered TCRs, and are essential for the effective recognition of specific pathogenic antigens among the huge repertoire of all antigens, which in turn is essential for the success of immunity against infection. Besides high concentration of a single molecule, as discussed above with actin or TCR, the vast majority of membraneless organells formed by phase separation consist of a collection of different macromolecules. The compartment of diverse molecules in a confined environment allow the membraneless organells to perform sophisticated biological functions. Super-enhancers are a paradigm model of transcriptional regulation where a large collection of transcriptional regulators are deposited in a short region to enable high level transcription. Recent studies demonstrated that phase separation is the underlying mechanism behind the formation of super-enhancers, where the collective action of transcriptional activators induce the condensates formation [9].

Given the essential roles of phase separation in regulating fundamental biological processes, it is not surprising that dysregulation of phase separation has emerged as novel causes of human diseases. A prominent example is Alzheimer's disease (AD). Although the mechanism of AD is not clear, several manifestations of the disease are clearly related to dysregulation phase separation. The hallmark of AD is the deposition of amyloid beta protein that leads to the formation of fibrillar amyloid plaques, a direct result of excessive amount of amyloid protein, and a likely consequence of dysregulated phase separation owing to the high concentration of amyloid proteins in disease neurons. Moreover, experimental data suggested that hyperphosphorylated tau protein leads to formation of neurofibrillary tangles inside nerve cell bodies, which eventually disintegrate microtubules, the structure essential for cell integrity and intracellular transportation, leading to another key characteristics of AD. In addition to AD, several other neurodegeneration diseases such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), also share a similar principle in disease characteristics with AD. Specifically, although the molecules involved in these diseases are different, they all characterized by

pathogenic aggregation of underlying molecules, indicating that dysregulation of phase separation is the common underlying mechanism in their pathogenesis.

PRINCIPLES GOVERNING THE FORMATION OF PHASE SEPARATION OF BIOLOGICAL MOLECULES

Strong evidence supports the idea that collective interaction among protein-protein and protein-RNA drives the liquid-liquid phase separation of macromolecules. The key underlying principle is that a network of interactions simultaneously occurs within a confined location. Consequently, domains in proteins or RNAs that facilitate protein-protein or protein-RNA interaction are the building block behind phase separation. A large number of proteins involved in phase separation are intrinsically disordered proteins (IDPs). Unlike classical protein that fold into well-defined 3-D structures, IDPs are characterized by low sequence complexity and typically do not have fixed structures. IDPs fold into defined structures upon protein-protein interaction and are critical in promoting phase separation. A prominent example is that IDPs share similar characteristics with prion proteins. These IDPs composed mostly of polar amino acids which promote protein-protein interaction and tends to form prion like aggregates in solution. In a similar fashion, another class of IDPs consists of a large number of charged amino acids. These IDPs have a high tendency to form multi-molecular complex through electrostatic interactions under suitable pH and ionic conditions. Besides IDPs, protein or RNA with multiple domain/regions mediating intra-molecular interactions are another key component to initiate phase separation. The central components in membraneless organells such as nucleolus, paraspeckles, snurposomes, are long RNA molecules. These RNAs contain multiple binding sites for protein components in the membraneless organells, and served as scaffold for the assembly of large protein-RNA complexes. On the other hand, multi-domain proteins serve an similar purpose as scaffold in mediating multi-molecule complex formation. These multi-domain proteins are especially enriched in signaling pathways and transcriptional regulatory machineries, where condensates formed by large protein complex play critical regulatory roles.

As an integrative mechanism of biological systems, phase separation of macromolecules is naturally under tight regulation. Although clear mechanisms have yet to be established, basic molecular mechanisms controlling protein and RNA metabolism and modification are natural candidates. Because protein concentration is the crucial parameters controlling membraneless organelles formation, molecular mechanisms controlling mRNA transcription and protein translation, and post-translational protein modification that could regulate protein half-life could readily induce changes in protein concentration, and subsequently induce phase separation. On the other hand, mechanism that changes protein affinity or valency could also regulate phase separation. For example, the clustering of TCRs are regulated by kinase that could phosphorylate intercellular domain of TCR proteins, which will substantially increase the TCR-TCR interaction affinity, leading to the formation of TCR clusters. Conversely, phosphatase that dephosphorylate TCR will disrupt the TCR cluster, terminating the hypersensitive signaling of the TCR pathways. Furthermore, the structures of membraneless organelles are also under dynamic regulation. For example, the structure of nucleolus displays regulated dynamics and the GC dissolves during mitosis but the FC and DFC components remains.

PHASE TRANSITION IN CELLULAR DIFFERENTIATION AND EMT

Beyond phase separation at molecular level, phase transition at cellular level is also a well-established phenome. The epithelial-to-mesenchymal transition (EMT) is a prominent example of phase transition. EMT is a cellular reprogramming process in which epithelial cells forming close contacts with their neighbors and displaying apicobasal polarity transit into mesenchymal cells with loose connections to extracellular matrix and lack of polarity [10–13]. EMT involves dramatic changes at both phenotypical and molecular levels [14]. The phenotypical changes during EMT are characterized by the remodeling of the epithelial adhesive junctions and cytoskeletons, resulting in increased motility and cells with invasive phenotype [10]. Epithelial cells can acquire mesenchymal characteristics that was first observed in primitive streak of chick embryos by Elizabeth Hay in 1980s [15]. Since then, EMT has been shown to play essential roles in diverse biological processes such as embryo development, gastrulation, organ development, wound healing, and tumorigenesis [10]. Classically, EMT has been regarded as a paradigm of cellular plasticity where cells shift between two alternative states, epithelial or mesenchymal. However, recent studies have demonstrated a greater flexibility and cells undergoing EMT transit through multiple intermediate states [13,16,17]. Moreover, these state transitions often display controlled reversibility and the associated dynamics has been implicated in key disease processes such as metastasis [18–22].

The changes in phenotype during EMT is accompanied by dramatic changes in gene expression programs. Hundreds of genes revert their expression in cells undergoing EMT [23–25]. Genes that are essential for

cellular junction and cellular polarity, such as E-cadherin, are downregulated and genes associated cellular motility such as fibronectin and N-cadherin, are dramatically upregulated. Although the full gene regulatory program controlling the massive changes in gene expression and phenotype during EMT is not fully understood, a regulatory theme consisting of cascading feedback loops formed between a few master transcriptional regulators and microRNAs has been established [26–28]. These feedback loops are capable of generating bi-stability and demonstrate switch like behavior, which captures essential characteristics of EMT transitions [29]. Critically, mathematical simulation and analysis of state transitions during EMT suggest that EMT resembles phase transitions in other fields [26,29–32], and the tipping point of a biological process, which signals the imminent critical transition, may be essential to fully understood the dynamics of EMT.

Cells undergoing EMT transit through a linear trajectory with epithelial and mesenchymal phenotypes as starting and ending states respectively. Despite this linearity, recent studies have revealed remarkable complexities associated EMT. Instead of a simple two state scenario, EMT often involves multiple distinct intermediate states. EMT demonstrates remarkable similarity to phase transition in physics, economics or ecology. Cells in the same state display homogeneous characteristics, and remarkably distinct after transit into a different state. Increasing evidence suggests that phase coexistence is common during EMT where cells in the process of EMT can simultaneously possess characteristics of epithelial and mesenchymal cells. Moreover, feedback loops are essential for the regulation of EMT and consequently, bi-stability and critical transitions have become a trademark of EMT [27].

REGULATORY NETWORKS GOVERNING TRANSITIONS DURING EMT

In the past few years it has become clear that EMT is regulated by several layers of regulatory networks [33]. The core EMT regulatory networks are dependent on master transcriptional factors (TFs) and microRNAs that form bi-directional negative regulation with these TFs [34]. The best characterized TFs driving EMT are SNAI1/2 and ZEB1/2 [35,36]. These TFs not only directly repress the hallmark epithelial gene CDH1 (E-cadherin), but also repress other junctional proteins that are responsible for cell-cell contact. Moreover, EMT driving TFs also promote the expression of mesenchymal genes such as FN1 and VIM. Interestingly, experimental results suggest that overexpressing SNAI1/2 or ZEB1/2 alone is sufficient to drive EMT, establishing their roles as master regulators of EMT [25,34]. Importantly, SNAIL1/2 also activate the expression of ZEB1/2, forming a positive activation cascade [27]. On top of EMT driven TFs, a crucial layer of regulation is formed by miRNAs. Double negative feedback loops are form between SNAIL1/2-miR-34 and ZEB1/2-miR-200s by reciprocally repressing each other [33,37–40]. These double negative feedback loops are crucial to establish bi-stability, which is a hallmark of phase transition, and prevent cells from uncontrolled overexpression of mesenchymal genes during EMT [26].

Besides SNAIL and ZEB family of TFs, more than 20 additional TFs have been established as EMT drivers in various cellular and tissue models of EMT [33]. Importantly, numerous TFs demonstrate significant expression changes in the same cellular model of EMT, suggesting that the EMT driving circuits is more complex than the canonical SNAI/ZEB model [23]. The large number of EMT driving TFs is consistent with the concept that cells undergoing EMT transit through multiple intermediate states beside epithelial and mesenchymal state. Although it is natural to extend the EMT regulatory circuits to include addition double negative feedback loops with new TFs to account for the additional states, the extension is not straightforward because some of the intermediate states are metastable, supporting a more delicate wiring of the regulatory circuits [13].

CRITICAL TRANSITIONS WITH THEIR TIPPING POINTS QUANTIFIED BY DYNAMIC NETWORK BIOMARKERS

Many biological processes including disease progression can be generally divided into three stages or states, *i.e.*, pre-transition state, critical state and post-transition state [2,3]. Specifically, the three states for EMT can be defined as E-state (epithelial state), critical state (tipping point) and M-state (mesenchymal state), where the critical state is the steady state just before the critical transition to M-state and is the tipping point of the EMT process. The state of a biological system at the critical state is still reversible to the E-state with appropriate perturbations, but becomes very difficult to return to E-state after the critical transition. Thus, how to quantify the critical state and further identify its driving molecules are crucial not only for revealing molecular mechanism of EMT but also for providing early-warning signals of this transition. However, it is a difficult task to detect the tipping point due to no significant difference between E-state and critical state, which makes the traditional methods or biomarkers fail.

To overcome such a problem, dynamic network biomarker (DNB) method [2,3,27–29,41–44] was developed to identify the critical state only based on the

measured omics data. Actually, mathematically, if a biological system is considered as a nonlinear dynamic system, the tipping point is a bifurcation point, which has a few important dynamic features (generic properties from mathematic viewpoint). Thus, by exploring both network information and dynamics information of the critical state, we can derive three necessary conditions of the criticality, which can be used to quantify DNB. DNB is actually a group of molecules with strong fluctuations and also high correlation, and can signal the tipping point even though there are no significant differences between E-state and critical state in terms of gene/protein expressions [2,3,27–29,41–44]. Since DNB theory is based mainly on the second order statistics (such as deviation and correlation) rather than the traditional first-order statistics (such as average values), we can even detect the "dark genes" in terms of gene expression, which have no differential expression changes during the EMT but play important roles from the perspectives of network [2,3,41–44]. DNB theory has been applied to various areas by many researchers, including successfully identifying the tipping points of cell fate decision [45], studying immune checkpoint blockade [46], and obtaining the early-warning signals of cancer metastasis [2,3,41–44] and detecting two tipping points of type-2 diabetes [47]. EMT is a typical phase transition process from E-state to M-state, and its bi-stability or multi-stability feature implies that there are tipping points just before the critical transitions, which play key roles for the phase transition. Thus, DNB can also be expected to apply to EMT for identifying its tipping points of the critical transitions, but how to accurately quantify the tipping points of EMT based on the observed data by DNB is a future topic. DNB is to provide the early-warning signals just before the critical transition or predict the EMT, while another concept exploring network information is network biomarker (NB) [38–40,48,49], which can identify the functional genes or molecules during the EMT from network viewpoint and thus can provide the functional annotation for the critical transitions or tipping points of EMT. Network biomarker measures the differential changes of networks (*e.g.*, differential correlations or associations between a pair of genes) before and after the EMT transition, which are completely different from the traditional molecular biomarkers measuring the differential expressions. Both DNB and NB are expected to provide the network insights into the molecular mechanisms of critical transitions and their tipping points for EMT in future.

PERSPECTIVES

Recent studies show that EMT is a complicated dynamic process where cells transit through multiple intermediate states or tipping points undergoing EMT from E-state to M-state. Experimental data and mathematical modeling have revealed complex regulatory networks underlying state transition during EMT to certain extent. Although there are significant advances on understanding EMT, many open questions and challenges remain in this fast moving field, *e.g.*, how to identify all major drivers or master regulators for the EMT; how to detect the tipping points during EMT or MET; what is the role of intermediate states during EMT; how MET process from M-state to E-state is related to EMT process. Recent rapid advances on high throughput technologies open a new way to study complex biological systems from a system-wide perspective, and are expected to elucidate the molecular mechanism of EMT at a system/network level, by exploring network information such as network biomarker (NB) [38–40,48,49] or by exploiting dynamical information [50] such as DNB (Figure 1) [2,3,29,41–44,47,51,52].

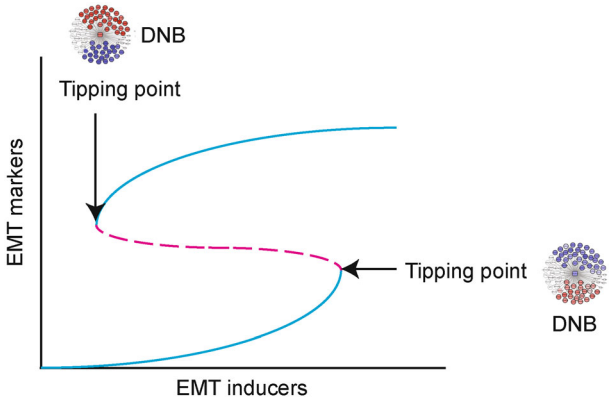

Figure 1. Graph illustrating the tipping points and corresponding dynamic network biomarkers associated with the bistable phenomena during EMT.

The intrinsic similarity between the characteristics of phase separation of macromolecules and state transition of biological networks suggests that there may be deep connections between these phenomena. For example, concentration is a fundamental parameter controlling macromolecule phase separation. Consequently, regulatory networks controlling the production, destruction, stability and motility of targeted molecules, could directly regulate phase separation. Conversely, functional consequence of macromolecule phase separation could also impact the dynamics of associated regulatory networks. For example, EMT genes are known to be driven by super-enhancers, which resulted from a high concentration of transcription factors within a small volume, a classical example of macromolecule phase separation. Thus, future studies investigating the

mechanistic connection between macromolecule phase separation and network state transition could reveal fundamental mechanisms underlying key biological processes.

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