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Review

A Novel Lens for Cell Volume Regulation: Liquid–Liquid Phase Separation

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Key Words
Cell volume sensing • Cell volume regulation • Osmotic stress response • Biomolecular condensates • Phase separation

Abstract
Cells are constantly exposed to the risk of volume perturbation under physiological conditions. The increase or decrease in cell volume accompanies intracellular changes in cell membrane tension, ionic strength/concentration and macromolecular crowding. To avoid deleterious consequences caused by cell volume perturbation, cells have volume recovery systems that regulate osmotic water flow by transporting ions and organic osmolytes across the cell membrane. Thus far, a number of biomolecules have been reported to regulate cell volume. However, the question of how cells sense volume change and modulate volume regulatory systems is not fully understood. Recently, the existence and significance of phase-separated biomolecular condensates have been revealed in numerous physiological events, including cell volume perturbation. In this review, we summarize the current understanding of cell volume-sensing mechanisms, introduce recent studies on biomolecular condensates induced by cell volume change and discuss how biomolecular condensates contribute to cell volume sensing and cell volume maintenance. In addition to previous studies of biochemistry, molecular biology and cell biology, a phase separation perspective will allow us to understand the complicated volume regulatory systems of cells.

Introduction

A number of anisotonic conditions are observed under both physiological and pathologic conditions. In the kidney, osmolarity gradation from juxtamedullary nephrons to the inner medulla is generated to concentrate urine [1]. Hence, kidney medulla cells and blood cells passing through the kidney are exposed to high medullary osmolarity [2]. Stomach and intestinal cells are also exposed to acute changes in osmolarity after food intake that induce anisotonicity [2]. In addition to physiological conditions, anisotonic conditions are generated in various injuries and diseases. Impaired water flow from the interstitial

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space to the capillary bed results in edema, which exposes interstitial cells to a hypotonic environment [3]. In the cancer microenvironment, extracellular osmolarity is changed because of the abnormal activity of ion channels and transporters on cancer cells and because of the release of metabolites, including lactic acid, from cancer cells [4–6]. Other examples of anisotonic conditions are found in [7].

The unequal osmolarity inside and outside cells (i.e., osmotic stress) causes cell volume perturbations because water-specific membrane channel proteins, aquaporins (AQPs), promote water molecules to permeate the cell membrane to a greater extent than other polar substances [8]. Cells shrink when the extracellular osmolarity is higher than the intracellular osmolarity (i.e., hyperosmotic stress), and cells swell when the extracellular osmolarity is lower than the intracellular osmolarity (i.e., hypoosmotic stress). Cell volume perturbation induced by osmotic stress causes severe stress in cells and disrupts cellular homeostasis. When the cell volume is decreased, the intracellular concentration of macromolecules, such as proteins, nucleic acids and lipids, is increased. The effects of hyperosmotic stress extend to DNA damage and cell cycle arrest [9]. In contrast, when the cell volume is increased, the intracellular concentration of macromolecules is decreased. Severe mechanical stretch under hypoosmotic stress changes the density of the lipid bilayer and the specific structures on the cell membrane and eventually results in cell burst [10].

Cells recover their physiological volume upon osmotic stress induction by transporting osmolytes (i.e., ions and organic compounds affecting osmolarity) and by synthesizing organic osmolytes [2]. However, the questions of how cells sense volume perturbation and trigger volume recovery systems are still under discussion. The previous systematic review [11] enables us to understand the cellular mechanisms for sensing cell volume perturbation in terms of three intracellular changes: 1) change in mechanical forces on membrane proteins, 2) change in intracellular ionic strength/concentration and 3) change in macromolecular crowding (Fig. 1). These changes trigger functional alterations, such as changes in membrane protein conformation, enzymatic activity and reaction equilibrium, followed by further effective cellular responses. Although these alterations have been interpreted mainly from the perspectives of biochemistry and molecular biology, recent studies [12–17] provide a spatiotemporal understanding of these alterations with biomolecular condensates induced by liquid–liquid phase separation (LLPS). Biomolecular condensates are membraneless but form phase-separated compartments of biomolecules that undergo dynamic and rapid phase transition according to the thermodynamic energetic states in the cell [18–20]. In other words, the states of biomolecular condensates are defined and altered based on the concentration and state of constituent biomolecules and environmental conditions, including the three intracellular changes, which is a reasonable mechanism for explaining cellular volume sensing. Biomolecular condensates and LLPS are currently attracting the attention of many researchers in a broad range of life sciences not only because their involvement in physiological events and diseases is suggested [21, 22] but also because the "lens" of LLPS may bridge the gap in the interpretations informed by molecular biology/biochemistry and cell biology.

In this review, we summarize the current understanding of cell volume regulation and cell volume sensing in the sections "*Cell volume regulation by volume effectors and upstream signaling molecules*" and "*Cell volume-sensing mechanisms induced via three intracellular changes*", respectively. Next, we briefly explain biomolecular condensates and LLPS in the section "*LLPS, a novel perspective for understanding cell volume-sensing mechanisms*". We then introduce recent studies about biomolecular condensates under cell volume perturbation and discuss the involvement of LLPS in cell volume sensing in the section "*Biomolecules acting as inherent sensors of cell volume through LLPS*". Finally, we discuss the potential of LLPS in other volume-changing events in the section "*LLPS potential in cellular volume-changing events*".

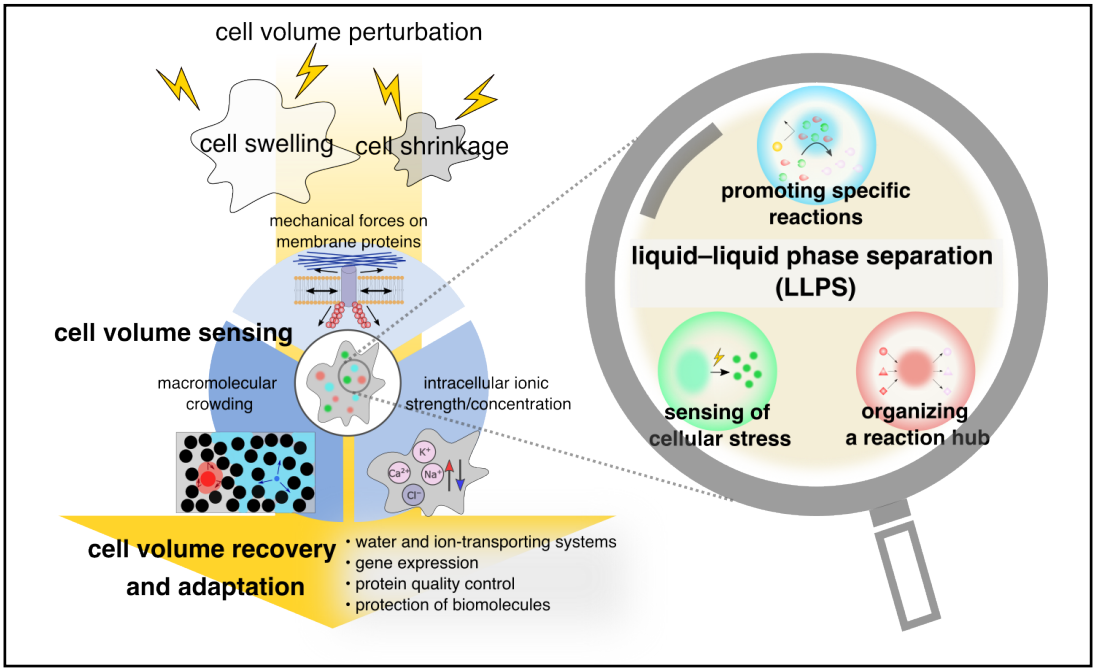

Fig. 1. LLPS involvement in cell volume sensing and regulation. Cell volume perturbation causes three intracellular changes: 1) changes in mechanical forces on membrane sensors, 2) changes in intracellular ionic strength/concentration and 3) changes in macromolecular crowding. These intracellular changes lead to effective cellular responses, including the direct and indirect coordination of water and ion-transporting systems, gene expression, protein quality control and protection of biomolecules for cell volume recovery and adaptation. Although these alterations have been interpreted mainly from the perspectives of biochemistry and molecular biology, recent studies provide a spatiotemporal understanding of these functional alterations with biomolecular condensates induced by liquid–liquid phase separation (LLPS). The "lens" of LLPS enables us to understand the complicated cell volume regulatory mechanism and break through gaps between cell biology and biochemistry/molecular biology interpretations.

Cell volume regulation by volume effectors and upstream signaling molecules

Cells regulate volume by altering the transportation of ions and osmolytes across the cell membrane. After cell shrinkage induced by hyperosmotic stress, cells recover their volume because of the rapid intake of ions, which is called regulatory volume increase (RVI) [11]. On the other hand, after swelling because of hypoosmotic stress, cells reduce their volume by the efflux of ions and organic osmolytes, a process called regulatory volume decrease (RVD) [11].

The regulation of RVI under hyperosmotic stress mainly depends on the Na^+/H^+ exchanger (NHE), Na^+-K^+-Cl^- cotransporter (NKCC) and hypertonicity-induced cation channel (HICC). NHEs regulate intracellular pH by enabling the 1:1 exchange of Na^+ and H^+ [23]. They are known to be activated by cell shrinkage under hyperosmotic stress, and the inhibition or depletion of NHEs suppresses RVI [24–27]. The ion exchanges by NHEs are generally coupled with Cl^-/HCO_3^- exchange by anion exchangers (AEs) [28, 29], and coupled ion exchange by NHEs and AEs is required for RVI [30–32]. NKCCs cotransport Na^+, K^+ and Cl^- into cells at a ratio of 1:1:2 [33]. NKCCs are also activated under hyperosmotic stress and required for RVI [34]. HICCs are defined based on electrophysiology and required for RVI in many types of cells [35–37]. Thus far, it has been reported that transient receptor potential (TRP) cation channels subfamily M members 2 and 5 (TRPM2 and TRPM5) and epithelial Na^+ channels (ENaCs) in HeLa or HepG2 cells show typical characteristics of HICCs [38–41]. However, the molecular identity of HICC has not yet been firmly established [42], and further continued molecular analyses for identifying HICCs are required.

The regulation of RVD after cell swelling critically depends on anionic efflux via volume-regulated anion channels (VRACs) because the basal Cl^- conductance is low and cationic efflux is limited by membrane potential unless the conductance of Cl^- is increased [11, 43, 44]. While its pharmacological and electrophysiological properties had been well characterized [11], the molecular identity of VRACs was not revealed despite decades of investigation [42]. In 2014, leucine-rich repeat-containing protein 8A (LRRC8A, also known as SWELL1) and other LRRC8 family members, LRRC8B–E, were reported to be the molecular identity of VRACs [45, 46], although bestrophin 1 is also suggested to be a VRAC in a few cell lines [47]. In addition to VRACs, K^+-Cl^- cotransporters (KCCs) are activated after cell swelling, and KCCs cotransport K^+ and Cl^- into the extracellular space at a ratio of 1:1 and contribute to the RVD process [48–51]. Ca^{2+}-activated K^+ channels are also involved in the RVD process by inducing increased K^+ efflux [52, 53]. Anion efflux during RVD must be coupled with cation to maintain electroneutrality [42], and Ca^{2+}-activated K^+ channels may also contribute to this maintenance. The influx of Ca^{2+} to activate Ca^{2+}-activated K^+ channels during RVD is possibly dependent on TRP channels, which contribute to RVD while promoting the influx of cations [54]. TRPV4 and TRPM7 contribute to Ca^{2+} influx and RVD [55, 56]. We note that the contributions of these ion channels and transporters to RVI and RVD vary among cell lines [35, 57].

In addition to their roles in RVI and RVD, these effector molecules are essential in cellular activities accompanied by cell volume changes, such as cell proliferation, cell migration and apoptosis [11, 58, 59]. During the early stage of apoptosis, cell volume is reduced by osmotic water efflux, which is called apoptotic volume decrease (AVD) [60, 61]. Similar to the process in RVD, Cl^- efflux accompanied by K^+ efflux mainly contributes to AVD [62]. The inhibition of Cl^- channels suppresses AVD and apoptotic cell death in various cell types [60, 61]. After LRRC8A was determined to be a critical VRAC component, the knockdown of LRRC8A was reported to suppress cisplatin-induced apoptosis [63]. In addition, the overexpression of LRRC8A promoted cisplatin-resistant cell death [63]. These studies indicate that VRAC/LRRC8 plays a central role in apoptosis and RVD and imply commonalities in cell volume regulation.

Cell volume effector molecules are regulated by numerous upstream signaling molecules [11]. Cation–chloride cotransporters (CCCs), including NKCCs, KCCs, and sodium–chloride cotransporters (NCCs), are regulated by with-no-lysine [K] (WNK) family proteins via the phosphorylation cascade [64]. The activity and phosphorylation of WNK1, WNK3, and WNK4 are altered by both hyperosmotic stress and hypoosmotic stress, although their direction of this modulation likely varies between cell types [65–67]. WNKs phosphorylate and activate STE20/SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress-responsive kinase 1 (OSR1) [64], which subsequently promote the phosphorylation of CCCs corresponding to NKCC and NCC activation and KCC inactivation [68, 69]. Indeed, the deletion of WNK1 results in the lack of NKCC1 activation and suppression of RVI [70]. Knocking down either WNK1 or OSR1 suppressed temozolomide-induced NKCC activation and glioma cell migration [71]. Knocking down WNK3 suppressed RVI [72], but one report indicated that this effect was limited to the early phase [73].

In addition to regulation via the WNKs-SPAK/OSR1 axis, stress-activated mitogen-activated protein kinases (MAPKs) show changed activity levels after cell volume perturbation and regulate cell volume effector molecules [74]. Extracellular signal-regulated kinase (ERK) and upstream protein kinase C (PKC)-δ activation mediates the NKCC activation in human tracheal epithelial cells [75]. p38 MAPK activity positively regulates HICC activation in HeLa cells [76]. p38 MAPK activity is also required for volume-regulated K^+ release in perfused rat liver, although the critical volume effector is not clear [77]. p38 activity and JNK activity are required for the activation of NHEs in Ehrlich ascites tumor (EAT) cells and *Xenopus* oocytes, respectively [78, 79]. The activity of the MAPK kinase MEK1 is required for the establishment of swelling-activated Cl^- current, probably via VRACs [80, 81]. While it is unclear how MAPKs alter the activity of volume effectors in most cases, it has been reported that ERK directly phosphorylates and activates the Kv4.2 K^+ channel [82], implying

a potential mechanism that MAPKs directly phosphorylate cell volume effectors to regulate their effector activities. Moreover, MAPKs regulate cell volume effectors at the expression level: the mRNA expression of AQP1 and AQP5 is dependent on ERK activation mediated by MAPK/ERK kinase 2 (MEKK2) [83]. Of note, membrane trafficking also contributes to the expression of ion channels and transporters on the cell membrane [84–86].

Small GTP-binding proteins also regulate ion channels and transporters, as inhibitors of Rho and Rho kinase are reported to reduce the swelling-activated Cl^- current through VRACs [87]. Small GTP-binding proteins regulate VRAC, possibly by altering the actin cytoskeleton as suggested by disruption of the F-actin cytoskeleton activating VRACs [88, 89]. A change in the level of cellular phosphatidylinositol 4,5-bisphosphate (PIP_2) also has an impact on the activity of volume effectors. The activity of ENaC increases upon PIP_2 directly binding to its cytoplasmic region [90, 91]. The local reduction in PIP_2 downregulates the activity of TRPM7 by phospholipase C (PLC) activation [92]. Excellent reviews have systematically covered cell volume effectors and upstream signaling molecules [2, 7, 11].

Cell volume-sensing mechanisms induced via three intracellular changes

Although numerous volume effectors and their upstream regulators have been identified in various cell types [11], the relationships among these molecules remain mostly obscure. In particular, the mechanism by which cells sense cell volume changes has been a long-standing and challenging enigma. As previously described by Hoffmann and colleagues [11], we can understand the cellular mechanisms for sensing cell volume perturbation in terms of three intracellular changes: 1) change in mechanical forces on membrane proteins, 2) change in intracellular ionic strength/concentration and 3) change in macromolecular crowding (Fig. 1). Throughout the long history of studies on cell volume regulation, the term "sensor" has been used vaguely, with a variety of definitions. In this review, we define sensor as a substantial sensor, including a single molecule, complex or assembly that primarily recognizes any of the triad changes and transduces the recognized signal to downstream molecules involved in the subsequent cellular responses. Here, we introduce examples of cell volume sensors and describe cell volume-sensing mechanisms via the triad from the perspectives of biochemistry and molecular biology.

Cell volume-sensing mechanisms induced via changes in mechanical forces on membrane sensors

As one mechanism, cells sense volume perturbation via changes in the cellular membrane. Here, we focus on membrane sensors that are directly affected by mechanical forces in the cell membrane. Of note, volume-sensitive phospholipases and phospholipid kinases may be activated or inactivated during cell volume perturbation, leading to changes in mechanical forces on the corresponding membrane sensor proteins by altering the membrane curvature or thickness [11]. In this case, phospholipases and phospholipid kinases or their upstream molecules are the bona fide sensors of cell volume perturbation.

Some ion channels are physically pulled or distorted by the surrounding lipid bilayer, and thus, their conformations are mechanically altered into open form to enable ion flow. In bacteria, the mechanosensitive channels MscL and MscS are activated by hypoosmotic stress and are necessary for survival in a hypoosmotic environment [93]. Compared to cells with rigid cell wall, such as prokaryotes and plant cells, animal cells have greater excess membrane areas, like ones in caveolae, that can buffer membrane stretching [10, 94]. Nevertheless, mechanosensitive channels in animal cells are activated via conformational changes induced by membrane stretching. Many TRP channels are known to be activated by cell membrane stretching [54]. TRPC1 is activated by the extension of the plasma membrane [95], and the TRPC1 knockdown reduces the rate of RVD after hypoosmotic cell swelling [96], suggesting that the mechanical activation of TRPC1 is necessary for RVD. Piezo1 and Piezo2 are also cationic channels that are mechanically activated [97]. They contribute to a

number of mechanotransduction processes [98], and they are suggested to play roles in the volume regulation of red blood cells [99, 100]; namely, mechanical stretch induces Piezo1-dependent Ca^{2+} entry in red blood cells, and the deletion of Piezo1 promotes hemolysis [99].

Some adhesion proteins and receptors on the cell membrane can sense changes in mechanical forces on them. Integrins are thought to sense the change in membrane tension. Cell swelling by hypoosmotic stress increases the active (high affinity) conformation of integrin [101]. In addition, the bonds formed between fibronectin and integrin generate mechanical force and change the conformation of integrin [102, 103]. These results imply a scenario that mechanical forces by membrane stretching and extracellular matrix (ECM)/cytoskeleton-mediated pulling directly change the conformation of integrin and increase its interaction with its ligands. The conformational change of integrin by membrane stretching leads to increased binding with the ECM, which is required for downstream JNK activation [104]. In addition, the activation of integrin regulates VRACs [105–107]; several K^+ channels, including Kv1.3 [108, 109], Kv4.2 and Kv1.4 [110]; and Ca^{2+}-dependent K^+ channels [111]. Growth factor receptors on the cell membrane may also function as cell volume sensors. It has been suggested that the decrease in tension during cell shrinkage causes the clustering and activation of epidermal growth factor (EGF) receptors in some cell types [112, 113]. In addition, EGF receptors on Swiss 3T3 fibroblasts and the ErbB4 EGF receptor on cerebellar granular neurons are activated by membrane stretching [114, 115], which regulates taurine efflux via the PI3K-PKB and MEK1/2-ERK1/2 pathways [114].

Caveolae are thought to sense the cell membrane tension changes by altering protein clustering. Caveolin-1, the main component of caveolae, specifically binds to G_q protein α subunits ($G\alpha_q$) and prolongs the active state of $G\alpha_q$ upon stimulation, which is required for PLCβ activation to produce 1,4,5-trisphosphate from PIP_2, leading to the release of Ca^{2+} from the endoplasmic reticulum [116–118]. Hypoosmotic stress deforms caveola by interrupting the interaction between caveolin-1 and cavin1 [10, 119]. The deformation of caveolae in stretched membranes decreases the caveolin-1–$G\alpha_q$ association and reduces Ca^{2+} signaling [119]. Since the change in Ca^{2+} concentration can affect cell volume via the activation of Ca^{2+}-dependent K^+ channels, the deformation of caveolae may regulate cell volume effectors.

These examples indicate that cells sense cell volume perturbation via changes in mechanical forces on membrane sensors, leading to cell volume regulation.

Cell volume-sensing mechanisms induced via changes in intracellular ionic strength/concentration

Cell swelling accompanies a decrease in ion concentration, and cell shrinking accompanies an increase in ion concentration. Altered ionic strength/concentration changes the electrostatic interactions and conformations of sensory channels and enzymes.

VRACs are activated by the reduction in ionic strength [120–122]. In reconstitution assays, purified LRRC8 proteins exhibited increased activity following the decrease in intracellular ionic strength [123]. While the LRRC8 activation mechanism is poorly understood, there are some clues. The structures of LRRC8A were resolved almost simultaneously by three groups [124–126]. When comparing the reported structures, the transmembrane domain of LRRC8A is packed more tightly at high ionic concentrations than at low ionic concentrations, raising a hypothetical simple mechanism that the ion strength-dependent conformational change affects channel gating [125]. The bacterial ABC transporter OpuA is activated via ion strength sensing [127]. As a molecular mechanism, it is considered that the increase in intracellular ion strength induce dissociation of a cation region in OpuA from anionic lipids in the cell membrane, followed by the conformational change and opening of the channel [128]. Similarly, a mammalian K^+ channel, TWIK-related K^+ channel-1 (TREK-1), interacts with anionic lipids via its intracellular cation-rich region, which is required for mechanical channel gating [129]. Therefore, it is possible that a decrease in ionic strength changes the electrostatic interaction between VRAC and anionic lipid, leading to VRAC gating [123]. In particular, the N-terminal region of LRRC8 is critical for channel gating, as indicated by mutagenesis assays [130] and a structural analysis of LRRC8D [131]. Nevertheless, the

activity of VRACs is not regulated only by a change in ionic strength; several modulators have been found to regulate VRAC activity [87, 107, 132]. Hence, further analyses of the whole regulatory mechanism of VRACs are needed to reveal the cell volume-sensing mechanism.

The kinase activity of WNK1 is altered due to the conformational change induced by intracellular Cl^- [133]; according to the structural and mutagenesis analyses, Cl^- binds to L369 and L371 residues in WNK1, which inhibits WNK1 autophosphorylation and stabilizes the inactive form of WNK1. WNK4 also senses the change in Cl^- concentration [134]. In this report, the Cl^--insensitive WNK4 knockin mice exhibited the suppressed NCC activation during hypokalemia-induced decreases in intracellular Cl^- concentration. These structural and physiological experiments suggest that changes in intracellular ionic strength/concentration alter enzymatic activity via conformational changes.

The level of cellular PIP_2 increases and that of phosphatidylinositol monophosphate (PIP) decreases in hypertonic media, with changes in the opposite direction in hypotonic media [135]. The change in lipid composition is not observed when the cell volume changes in isotonic media with altered K^+ concentrations, suggesting that the decrease in ionic strength, not the cell swelling itself, alters the lipid composition. The change in lipid composition is basically induced by changes in phosphoinositide (PI)-specific phosphatase and kinase activities. These lines of outcomes may be integrated into a simple hypothesis that PI phosphatase and kinase sense the change in intracellular ionic strength/concentration and alter their enzymatic activities.

Combining these examples, cells sense cell volume perturbation via changes in intracellular ionic strength/concentration, leading to cell volume regulation. Similar to the excellent work on WNK1 [133], structure-based studies would deepen our understanding of the molecular mechanism.

Cell volume-sensing mechanisms induced via changes in macromolecular crowding

Macromolecular crowding is a condition where macromolecules occupy a high fraction of volume [136]. Hence, macromolecular crowding affects the macromolecular movement, association/dissociation rates and structure via excluded volume effect (see details in other excellent reviews [136–138]). Cells contain a significant fraction (typically 20–30%) of macromolecules in the cytosol [139], and the cell membrane is impermeable to macromolecules. Hence, cell volume perturbation alters intracellular macromolecular crowding as discussed in [140].

Based on computational analysis, changes in molecular crowding are predicted to alter protein conformation, reaction rate, and reaction equilibrium [136–138]. For instance, it has been predicted that the folded compact state of protein is stabilized in crowded media [141]. It is also predicted that the binding of macromolecules to other molecules and to cell membrane surface sites increases in crowded media [137, 140]. There are some reports that support these predictions experimentally *in vitro*. The melting temperature of yeast phosphoglycerate kinase (PGK) and white-egg lysozyme is increased by adding the crowding-inducing agent Ficoll or dextran [142, 143], indicating the conformational stabilization of proteins in crowded media. By adding Ficoll, the oligomerization of bovine pancreatic trypsin inhibitor is enhanced [144] while the self-assembly of HIV capsid protein is reduced [145], suggesting the effect of macromolecular crowding on the binding–unbinding reaction equilibrium. The chemical reaction rate is also affected by the change in diffusion and the affinity of the reactants; the activity of hexokinase is reduced by high concentrations of BSA [146]. Of note, the reaction rate and equilibrium point are also changed by altered protein concentration induced by cell volume perturbation. Other examples showing the effect of macromolecular crowding *in vitro* are reviewed in [137].

Several cell-based experiments suggest the effects of macromolecular crowding on volume effectors. NHE activity is high in resealed ghost erythrocytes with high protein concentrations [147], which is discussed in detail in [148]. It has been suggested that the addition of urea reduces macromolecular crowding without changing cell volume because attractive interactions between urea and cytoplasmic proteins reduce the thermodynamic

activity of cytoplasmic proteins [149–151]. Urea stimulates KCCs without changing cell volume in dog red blood cells [152], implying that the change in macromolecular crowding induced by urea affects KCC activity. These reports indicate that macromolecular crowding affects cell volume effectors. We note that changes in the activity of several enzymes induced by increased macromolecular crowding are suggested to activate NHE [147], indicating that macromolecular crowding-induced changes in multiple sensory molecules affect these cell volume effectors. Further details about cellular volume sensing and regulation via macromolecular crowding have been reviewed elsewhere: e.g., [151, 153].

LLPS, a novel perspective for understanding cell volume-sensing mechanisms

As described thus far, cells sense the three intracellular changes induced by cell volume perturbation to induce proper cellular responses. Considering recent studies [12–17], we can further understand these changes from the paradigm of biomolecular condensates induced by LLPS: liquid–liquid phase separation. LLPS is a physicochemical process in which "supersaturated solution of components spontaneously separates into two phases, a dense phase and a dilute phase" [20]. When the concentration of biomolecules is low and the interactions between macromolecules are weak, the molecules distribute homogenously in the cytosol. However, when the concentration of biomolecules exceeds the saturation threshold (C_{sat}), they separate into a dense phase with high concentration and a dilute phase with low concentration. C_{sat} is determined by the balance of associations between macromolecule and macromolecule and between macromolecule and water [18]. The dense phase structures induced via LLPS exhibit usually spherical shape with a variety of sizes (from nanometers to micrometers). They contain multiple components and possess diverse characteristics (e.g., speed or time of formation, composition and localization) depending on the property of each biomolecule (e.g., structure, amino acid sequence and hydrophilicity). For more details about the size behavior and other characteristics, see [18–20, 154, 155]. These structures are often expressed in different terms, including condensates, droplets, puncta, granules and aggregates. Following the current trend, we use the term biomolecular condensates in this review to emphasize their important feature: the structure to concentrate biomolecules within them [18–20]. Liquidity of biomolecular condensates can be examined by checking the condensate sphericity and the fusion of condensates over time *in vitro* and in cell, for example. Importantly, biomolecules within liquid phase can exchange between inside and outside the condensates, as often validated by fluorescence recovery after photobleaching (FRAP) experiments. One can also assess whether the condensates are liquid phase or solid phase by evaluating the reversibility of condensates by operating LLPS inducers (e.g., recovery from stress conditions, removal of post-translational modification).

According to thermodynamics, the most stable state in phase separation is assumed to be the state of a single condensate [156, 157]. However, when the speed in approaching this stable state is sufficiently slow, several small condensates are formed in a quasi-stable state [158]. These behaviors during phase separation depend on the system parameters including the following parameters changed by cell volume perturbation: temperature, ion strength/concentration, macromolecular crowding, and biomolecular state and concentration. For instance, condensate formation of SPD-5, a key scaffolding protein in pericentriolar material, is promoted *in vitro* when the concentration of the crowding agent polyethylene glycol (PEG) increases [159]. In this report, PEG concentration changes of only a few percentage points (every 1% change from 3% to 12%) resulted in a large difference in the amount of condensate, implying that the induction of LLPS is sufficiently sensitive to cell volume perturbation. To correctly interpret the studies using crowding agents, we note that inert polymers do not fully mimic the cytosolic depletion effects [160] and that PEG and other crowding agents should be used around the range of intracellular macromolecular density (e.g., 200–300 mg/mL in *E. coli*) [139]. Additionally, size of nucleoli, which are one of biomolecular condensates, negatively correlates with cell size when the number of nucleolar components is

fixed [161], suggesting that the decrease in cell volume induces phase separation by increasing biomolecular concentration. Changes in ionic strength/concentration affect phase separation when condensate formation is highly dependent on electrostatic interactions [162]. Nevertheless, while the physicochemical theories of biomolecular condensates agree with *in silico* and *in vitro* experiments, the cellular system is still disconnected from classic thermodynamic equilibrium since intracellular conditions are intrinsically dynamic; and cell-based investigations are ongoing in the context of LLPS.

While the unusual formation and phase transition of biomolecular condensates are considered as the important drivers for several diseases [21, 22], the LLPS-induced biomolecular condensates have a variety of functions by creating separate environments and spatiotemporally controlling specific biomolecules. Here, we leave the comprehensive categorizations of these functions to other reviews [18–20, 154] and introduce three examples among them (Fig. 1). First, biomolecular condensates function as sensing machinery for various cellular stresses. Under oxidative stress, the yeast ataxin-2 paralog Pbp1 forms condensates to inhibit target of rapamycin complex 1 (TORC1) and induce autophagy [163, 164]. This condensate formation of Pbp1 under oxidative stress is dependent on the oxidation of a methionine-rich low complexity region (LCR); that is, Pbp1 chemically senses oxidative stress via a redox reaction [163]. When exposed to pH stress, the yeast prion protein Sup35, a translation termination factor, forms condensates to protect itself from stress-induced damage [165]. Sup35 condensate formation under low pH depends on an acidic residue-enriched region; that is, Sup35 chemically senses pH changes via protonation [165]. In addition to pH stress, heat shock induces phase separation of the yeast poly(A)-binding protein Pab1 to maintain cell survival [166]. The formation of Pab1 condensates under high temperature is promoted by a proline-enriched LCR in Pab1 that exhibits hydrophobicity-dependent compaction [166]; that is, although generally seeming to be disadvantageous for condensation, high temperatures expand the conformation of the Pab1 LCR and induces Pab1 intramolecular interactions. Intriguingly, all these biomolecular condensates function as "sensors" for physicochemical stress, which is reasonably detected by phase separation/transition. Hence, from the perspective of LLPS, further theoretical understanding of the physicochemical cellular stress responses is getting elucidated. Second, biomolecular condensates promote specific reactions by selectively concentrating and excluding specific enzymes and reactants. During DNA damage, biomolecular condensates are formed at the damage site to concentrate DNA repairing enzymes and to protect DNA from further digestion [167, 168]. We note that hyperosmotic stress inhibits ionizing radiation (IR)-induced 53BP1 condensates [167], implying that cell volume changes affect the phase transition. Third, biomolecular condensates organize a reaction hub. The phase separation propensity of fragile X mental retardation protein (FMRP) and cell cycle associated protein 1 (CAPRIN1) with RNA is changed by their phosphorylation state and protein composition [169]. This selective and controlled phase separation modulates deadenylation and translation rates, suggesting a function for biomolecular condensates in the organization of a reaction hub. Heterochromatin protein 1a (HP1a) forms condensates with histone H3 and DNA [170–172], which may exclude or concentrate specific transcriptional factors and RNA polymerases to regulate gene expression by functioning as an organized reaction hub.

Biomolecules acting as inherent sensors of cell volume through LLPS

In addition to the versatile functions of biomolecular condensates described in the previous section, recent studies have demonstrated that biomolecular condensates serve as cell volume sensors; that is, cells sense the three aforementioned intracellular changes during cell volume perturbation via biomolecular phase separation or transition (Fig. 1). In this section, we introduce cell volume-sensing biomolecular condensates (Table 1) and discuss their physiological significance.

Table 1. Example of biomolecules that undergo phase separation during cell volume perturbation. *Human Genome Organisation (HUGO) gene symbol is written in parenthesis. †Concrete stimuli or time used in the reference are written in parenthesis. ‡Key supporting experiments are written in parenthesis. The single "?" sign in a field indicates no available information. Abbreviations: ANKRD52, ankyrin repeat domain 52; ASK3, apoptosis signal-regulating kinase 3; Avo3, a subunit of TORC2; CC, coiled-coil; CCC, cation–chloride cotransporter; CPSF6, cleavage and polyadenylation-specific factor 6; CT, C-terminal; DCP1A, decapping mRNA 1A; FCM, fluorescence confocal microscope; FRAP, fluorescence recovery after photobleaching; G3BP1, Ras GTPase-activating protein-binding protein 1; GFP, green fluorescent protein; GP value, generalized polarization value that reflect the overall organization of the plasma membrane; Hex, 1,6-hexanediol; Hsp, heat shock protein; IF, immunofluorescence; K48Ub, lysine 48-linked polyubiquitin; LATS, large tumor suppressor; LCR, low complexity region; LLPS, liquid–liquid phase separation; NLK, nemo-like kinase; OSR1, oxidative stress-responsive kinase 1; PABP, polyadenylate-binding protein; PalmC, a small-molecule tool that lowers cell membrane tension; PB, P-body; PES, PIP2-enriched structure; PIP2, phosphatidylinositol 4,5-bisphosphatate; PLD, prion like domain; PP6, protein phosphatase 6; PM, plasma membrane; PQC, protein quality control; RAD23B, RAD23 homolog B, nucleotide excision repair protein; Ref., reference; RNAPII, RNA polymerase II; Slm1, a downstream regulator of TORC2 signaling pathway; SPAK, STE20/SPS1-related proline/alanine-rich kinase; SG, stress granule; TAZ, transcriptional coactivator with PDZ-binding motif; TDP-43, transactive response (TAR) DNA-binding protein of 43 kDa; TEAD1, TEA domain transcription factor 1; TEM, transmission electron microscope; UBA domain, ubiquitin-associated domain; WNK, with-no-lysine [K] kinase; Y residue, tyrosine residue; YAP, yes-associated protein

Molecule*	Function of molecule	Condition of condensate formation†	Characteristics of biomolecular condensates‡	Suggested function of condensates	Remark for condensates	Ref.
Osmoresponsive kinase						
ASK3 (MAP3K15)	Cell volume regulator that negatively regulates the WNK1-SPAK/OSR1 pathway	Hyperosmotic stress (<10 sec) – 400, 500, 600, 700 mOsm (mannitol) – 400, 500, 600 mOsm (NaCl)	• Induced by increased macromolecular crowding (in vitro FCM and in silico modeling) • Liquid-like (FRAP in cells) • Membraneless (TEM)	• Acceleration of PP6-dependent dephosphorylation of ASK3 under hyperosmotic stress	• Necessary for CT CC domain and LCR. • Partly colocalized with ANKRD52 condensates at the merging area of condensates.	[17]
WNK1	Cell volume regulator that activates SPAK/OSR1	Hyperosmotic stress (<30 sec) – 500, 800 mOsm (sorbitol)	• Liquid-like (FRAP in cells)	• Activation of downstream SPAK and OSR1?	• Colocalized with SPAK and OSR1 partially. • CT noncatalytic region is sufficient. • Colocalized with WNK4 condensates. • No study has mentioned LLPS.	[65, 178, 179]
Transcriptional regulator						
YAP (YAP1)	Transcriptional coactivator with TAZ that plays a central role in the Hippo pathway	Hyperosmotic stress (<20 sec) – 0.2 M sorbitol supplementation	• Induced by increased macromolecular crowding (in vitro turbidity and FCM) • Liquid-like (FRAP in cells)	• Active transcriptional sites in the nucleus • Action sites of phosphorylation to regulate nuclear translocation of YAP in the cytosol	• Colocalized with TEAD and TAZ in nucleus. • Colocalized with LATS and NLK in cytosol. • RNAPII localizes at the surface of nuclear condensates within 2 hours.	[15]
CPSF6	Component of CPSFs-containing complex, which mediates transcriptional termination at 3′ end	Hypertonic stress (<1 min) – 300 mM Na^+ culture medium with 1/10 PBS	?	• Sequestration of CPSF6 to impair transcription termination	• Identified by a small-scale high-throughput IF screening.	[188]
PQC-related protein						
PSMB2	Core particle subunit of proteasome	Hyperosmotic stress (<10 sec) – 0.1, 0.2, 0.3 M sucrose or 50, 100 mM NaCl supplementation Hyperosmotic stress? (<5 min) – 0.2 M glucose supplementation	• Liquid-like (FRAP in cells) • Membraneless (TEM)	• Degradation of ribosomal proteins	• Colocalized with K48Ub, several ribosomal proteins, p97, RAD23B and UBE3A. • Another core subunit PSMA1 and a regulatory subunit PSMD6 also form condensates. • Reduced by Hex.	[16]
RAD23B	Substrate shuttling protein	Hyperosmotic stress (<30 min) – 0.2M sucrose supplementation	• Induced by increased macromolecular crowding and increased ionic concentration (in vitro FCM) • Liquid-like (FRAP in vitro)	• Induction of the LLPS of proteasome foci	• Multivalent hydrophobic interactions between two UBA domains and ≥4 length chain of K48Ub drives LLPS. • Necessary for the formation of PSMB2 condensates.	[16]
Hsp26, Hsp42, Hsp104, Ssa1, Ssa2, Ydj1, Sis1	Chaperone in yeast cells	Hyperosmotic stress (<10 sec) – direct addition of final 1, 1.5 M KCl to culture medium –direct addition of final 2 M sorbitol to culture medium for Hsp42, Hsp104, Ssa1, Ydj1	?	?	• Identified by GFP-fusion screening in yeast (Hsp26, Hsp42 and Sis1)	[227]
RNA binding protein						
TDP-43, PABP, G3BP1	RNA-binding proteins associated with SGs	Hyperosmotic stress (<1 min) – 600, 675, 700 mOsm culture (sorbitol) – 700 mOsm (NaCl)	• Probably liquid-like (FRAP in different stimulations)	• Sequestration to reduce translational activity • Protection of biomolecules from stress	• Colocalized with various translational factors.	[12, 13, 202, 228]
DCP1A	mRNA-decapping enzyme	Hyperosmotic stress (<10 sec) – 180, 210, 240, 300, 450 mM Na^+ culture medium with 10% PBS – 300 mOsm (sorbitol or sucrose) Hypertonic stress? – 90 mM Mg^{2+} or 180 mM Ca^{2+} culture medium with 10% PBS	?	?	• Different from conventional PBs. • Dependent on trimerization domain.	[188]
Membrane lipid						
PIP_2	Component of membrane lipids	Decreased in PM tension (<30 sec) – direct addition of final 1, 10 μM PalmC Hyperosmotic stress (<1 min) – direct addition of final 1 M sorbitol to culture medium	• Spontaneously induced after decreased PM tension via redistribution of pre-existing PIP_2 not via synthesis (GP value with Laurdan dye; ATP depletion)	• PES formation to inactivate TORC2 by clustering, to suppress synthesis of sphingolipids and to contribute to PM homeostasis	• $PH^{PLCδ}$ was used to detect the localization of PIP_2. • Colocalized with Avo3 and Slm1.	[14]
Else						
FLOE1	Uncharacterized *Arabidopsis thaliana* prion-like protein	Hydration (<10 sec) – submerged in water vs. glycerin – 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2 M NaCl solution –0.95 M mannitol solution or 0.725, 1.45 M sorbitol solution	• Liquid-like (FRAP in human cells) • Membraneless (TEM)	• Water stress sensing to inhibit germination under stress environment	• Necessary for Tyr residues in PLDs in both tobacco and U2OS cells.	[229]
Gln1	Glutamine synthetase in yeast cells	Hyperosmotic stress (<2–3 sec?) – direct addition of final 1 M KCl to culture medium	?	?	• Identified by GFP-fusion screening in yeast. • No upstream molecule controlling LLPS was obtained by genetic screening, suggesting control under physicochemical rule.	[227]
Tps1	Trehalose synthesis during severe stress in yeast cells					

Osmoresponsive kinases

Apoptosis signal-regulating kinase 3 (ASK3) is a unique osmoresponsive kinase; i.e., ASK3 is phosphorylated (activated) and dephosphorylated (inactivated) within only a few minutes of hypoosmotic stress and hyperosmotic stress induction, respectively, indicating that ASK3 activity is rapidly, reversibly and bidirectionally altered in response to osmotic stress [173]. In fact, ASK3 is required for RVD under hypoosmotic stress, while the direct dephosphorylation of ASK3 by protein phosphatase 6 (PP6) is necessary for RVI under hyperosmotic stress, demonstrating that ASK3 is an osmoresponsive kinase that orchestrates cell volume regulation [174]. Furthermore, ASK3 negatively regulates the WNK1-SPAK/OSR1 pathway in a kinase activity-dependent manner; the hyperactivation of SPAK/OSR1 and NCC is observed in the kidney of ASK3-knockout (KO) mice; ASK3-KO mice exhibit a hypertensive phenotype with aging or when fed a high-salt diet; these findings indicate that ASK3 regulates blood pressure via the downregulation of the WNK1-SPAK/OSR1 pathway in the kidney [173]. Based on the importance of ASK3 in osmoregulation, we conceived an experimental model of ASK3 regulation under osmotic stress to clarify a general cell volume-sensing mechanism, and we recently reported that ASK3 forms condensates in cells via LLPS immediately upon hyperosmotic stress induction [17]. Both *in vitro* and *in silico* experiments demonstrated that increased macromolecular crowding, not increased ion strength/concentration, is a driving force for the condensate formation of ASK3: the addition of Ficoll or PEG, but not NaCl, induced purified ASK3 condensate formation *in vitro*, and the existence of macromolecules in a simple computational model explained the characteristics of ASK3 condensates. In addition to the time-scale difference between condensate formation and ASK3 inactivation, ASK3 mutants lacking the ability to form condensates are not inactivated under hyperosmotic stress, while ASK3 normally forms condensates under hyperosmotic stress even when ASK3 inactivation is inhibited by PP6 knockdown, suggesting that ASK3 condensates are necessary for ASK3 inactivation under hyperosmotic stress. Furthermore, ASK3 condensates partly colocalize with PP6 condensates at the merging area of condensates, implying that ASK3 condensates provide PP6 with action sites for dephosphorylation within the limited space under hyperosmotic stress conditions. Therefore, it is considered that ASK3 condensates function as cell volume sensors to coordinate cell volume regulation.

Based on this example of ASK3 condensates, we should re-examine the "elucidated" molecular mechanisms from the perspective of phase separation/transition. For instance, WNK family proteins form "puncta" or "WNK bodies" in cultured cells and distal convoluted tubules [175–177]. The formation of WNK1 puncta by hyperosmotic stress was first reported in 2007 [65]. WNK1 puncta colocalize with OSR1, suggesting that WNK1 activates downstream signaling molecules in the puncta [178]. The formation of WNK bodies is also observed *in vivo*, and the physiological significance of WNK bodies has been proposed [179]. The WNK1 puncta/bodies basically seem to be spherical shape, dynamically move around the cytosol, disappear upon reversion to isoosmotic conditions and exhibit FRAP [65, 178, 179], indicating that WNK1 puncta/bodies have liquid properties and that WNK1 is exchanged between the dense phase and the dilute phase. Therefore, although not previously stated, WNK puncta or bodies can be understood as WNK condensates induced by LLPS, which would advance our understanding of WNK regulation. Interestingly, we observed that WNK1 condensates not completely colocalized but contacted with ASK3 condensates under hyperosmotic stress (our unpublished data). Considering that ASK3 negatively regulates WNK1 [173], the phase boundaries between ASK3 and WNK1 condensates may be involved in the ASK3-dependent downregulation of WNK1. In addition to ASK3 and WNKs, protein kinase N1 (PKN1) and PKN2 form vesicular structures under hyperosmotic stress [180], which may be the biomolecular condensates induced by phase separation.

Transcriptional regulators

Yes-associated protein (YAP) and its paralog, transcriptional coactivator with PDZ-binding motif (TAZ), are transcriptional coactivators that play a central role in the Hippo pathway regulating cell proliferation and cell death [181]. Recently, it was revealed that YAP

forms LLPS-induced condensates in both the nucleus and cytoplasm under hyperosmotic stress [15]. Purified YAP forms condensates *in vitro* when macromolecular crowding is increased by exposure to several crowding reagents, while changes in ion concentration do not trigger the *in vitro* condensate formation of YAP, suggesting that increased macromolecular crowding, not increased ion strength/concentration, is a driving force for YAP condensate formation, similar to ASK3 condensate formation. YAP condensates in the nucleus are enriched with the transcriptional factor TEAD1 and coactivator TAZ. In addition, nuclear YAP condensates localize at accessible chromatin domains, and RNA polymerase II localizes at the surface of YAP condensates within 2 hours after hyperosmotic stress induction, suggesting that the nuclear YAP condensates are active transcriptional sites. YAP condensates in the cytoplasm coordinate YAP translocation to the nucleus via phosphorylation regulation [15]. The S127 residue of YAP is phosphorylated by large tumor suppressor 1/2 (LATS1/2), and then, the phosphorylated YAP forms a tight association with 14-3-3 proteins and are retained in the cytoplasm [182, 183]. On the other hand, the S128 residue of YAP is phosphorylated by nemo-like kinase (NLK), which causes its dissociation from 14-3-3 proteins and its subsequent nuclear translocation [184, 185]. The cytoplasmic YAP condensates contain both LATS1 and NLK, and the S127A mutant and the S128A mutant of YAP show the increased and decreased nuclear translocation, respectively, under hyperosmotic stress with maintaining condensate-forming ability [15], implying that the cytoplasmic YAP condensates are the active sites for phosphorylation, which regulates the nuclear translocation of YAP. Transcriptional activation by TAZ is also regulated by phase separation [186]; however, its regulation during cell volume perturbation remains unclear. Therefore, it is implied that YAP and TAZ condensates function as cell volume sensors to modify gene expression during adaptation to hyperosmotic stress conditions.

Cleavage and polyadenylation-specific factor 6 (CPSF6) is a component of the CPSF-containing complex, which mediates transcriptional termination at the 3' end [187]. A small-scale high-throughput immunofluorescence screen revealed that CPSF6 undergoes phase separation during hyperosmotic stress [188]. The formation of CPSF6 condensates associated with the impairment of transcriptional termination, suggesting that CPSF6 is sequestered into condensates under hyperosmotic stress to alter downstream transcription [188]. Investigations into the involvement of macromolecular crowding and ion strength/concentration in the condensate formation of CPSF6 may reveal CPSF6 as a cell volume sensor that modulates gene expression in hyperosmotic stress adaptation.

Proteasomes

The proteasome is a large protein complex that degrades ubiquitin-tagged proteins for proteolysis and plays a major role in cellular protein quality control (PQC) in concert with molecular chaperones and autophagy [189–193]. In addition to the involvements of biomolecular condensates in chaperone proteins and autophagy [194–196], a recent study discovered that biomolecular condensates contribute to PQC via proteasome regulation: proteasome-containing nuclear foci, which contain and degrade ubiquitylated ribosomal proteins, are induced by LLPS under hyperosmotic stress [16]. A substrate-shuttling protein RAD23B and ubiquitylated proteins are required for the formation of proteasome foci. Purified RAD23B and K48-linked polyubiquitin (K48Ub) chains undergo co-phase separation *in vitro* when the crowding reagent or salt concentration is increased, implying that increased macromolecular crowding and/or ion strength/concentration trigger proteasome foci under hyperosmotic stress. Of note, the driving forces of proteasome foci generation are the multivalent hydrophobic interactions between two ubiquitin-associated (UBA) domains of RAD23B and K48Ub chains composed of more than four ubiquitin molecules, which can be affected by both macromolecular crowding and ion strength/concentration. Since ribosomal proteins have many disordered regions [197], they are easily denatured and form irreversible aggregates under hyperosmotic stress. Aggregation is caused by changes in noncovalent intramolecular or intermolecular interactions and loss of native conformation. A computational analysis predicted that the excluded volume promotes protein

aggregation [141], and the increased aggregation rate in crowded medium was observed experimentally [198]. Additionally, aggregation is caused by changes in ionic strength/concentration [199]. The denaturation and aggregation of proteins are often harmful to cells because they enfold and aggregate normally folded proteins and thus prevent the proper physiological functions of biomolecules [200, 201]. Therefore, it is implied that proteasome foci function as cell volume sensors to maintain proteostasis during adaptation to hyperosmotic stress.

RNP granules

Stress granules (SGs) are formed under hyperosmotic stress [12, 13, 202], and many kinds of RNA-binding proteins (RNPs) are exchanged between the inside and outside of SGs [202, 203], indicating that SGs are formed via LLPS under hyperosmotic stress. SGs sequester biomolecules required for translation, including mRNA, translation initiation factors and 40S ribosomal subunits [204]. As mentioned in the previous subsection, cell volume perturbation can induce the misfolding of proteins. The reduction in translational activity due to SG formation reduces the burden on proteolytic systems and contributes to PQC. Additionally, SGs contain signaling molecules, and the SG composition varies according to the induced stresses [205]. Hence, SGs act as suppressors or facilitators of specific reactions by sequestering or concentrating signaling molecules. SGs may also be essential for storing and protecting mRNA from severe cytosolic conditions since cell volume perturbation damages nucleic acids by changing the ionic strength/concentration [206]. Such a function of biomolecular condensates to protect cell-adapting biomolecules is also reported under nutrient-deprivation stress [165, 166]. Therefore, SGs under hyperosmotic stress are considered to function as cell volume sensors to coordinate hyperosmotic stress adaptation activities. Some regulatory mechanisms of SG formation and characteristics are suggested. For instance, it is suggested that the formation of SGs is induced by the depletion of ATP [207] which can act as a hydrotrope to solubilize hydrophobic proteins and deter their condensate formation [208]. This implies that energy depletion induces the formation of SGs. It is also reported that not only ATP itself but also various ATPases differently regulate interactions between biomolecules and SG assembly [209]. It seems that such a differentiated regulation finetune the roles of SGs for diverse stresses. Therefore, the mechanism of SG formation may reflect on the required adaptation depending on types of stress, and further investigations would give us the connective and comprehensive understanding on the functions of SGs during cell volume perturbation.

Related to SGs, it has been recently reported that decapping mRNA 1A (DCP1A), a component of processing bodies (PBs), forms condensates by osmotic cell shrinkage [188]. Although the material properties of DCP1A condensates were not investigated in this study, the hyperosmotic stress-induced DCP1A condensates melted when isoosmotic conditions were reestablished, suggesting that the DCP1A condensates are not mere aggregations. However, DCP1A condensates do not colocalize with several PB markers, implying that the physiological significance of DCP1A condensates is different from that of conventional PBs and is currently unknown [188]. We note that the authors utilized hyperosmotic stress as a macromolecular crowding inducer in this study, but whether increased macromolecular crowding and/or ion strength/concentration drives DCP1A condensates remains to be clarified. Hence, further studies may identify noncanonical DCP1A condensates as cell volume sensors.

Membrane lipids

In addition to proteins, membrane lipids are biomolecules that undergo phase separation depending on the saturation level of acyl chains; that is, saturated lipids tend to enter a liquid-ordered phase with highly ordered acyl chains, and unsaturated lipids tend to enter the liquid-disordered phase with disordered acyl chains [210]. The decrease in plasma membrane tension under hyperosmotic stress induces PIP_2 phase separation in the membrane, leading to cluster formation and inactivation of TORC2 in yeast [14].

TORC2 activation promotes the synthesis of sphingolipids by activating the direct TORC2 substrate Ypk1 [211], contributing to membrane tension homeostasis. Although the cell membrane tension differs between yeast and animal cells, the system in which membrane tension changes the activity of TORCs is conserved in mammalian cells [212]. These reports raise the possibility that the perspective of lipid LLPS helps us further understand the change in mechanical forces on membrane proteins under cell volume perturbation.

In this section, we described the biomolecular condensates in cell volume reduction. However, the phase transition of biomolecular condensates may occur not only in cell shrinkage but also in cell swelling, which diminishes the biomolecular condensates observed in the steady state or alters their material properties. Indeed, there are several well-known biomolecular condensates in steady state, such as nucleoli, nuclear speckles, Cajal bodies and P granules, but we may currently underestimate the existence of biomolecular condensates in the steady state. The biomolecule concentration required for condensate formation, namely, C_{sat}, can apparently vary due to the differences and stochastic fluctuations in local microenvironments. In fact, the induction of localized oligomerization enables biomolecules to undergo localized phase separation far below global C_{sat}, indicating that even biomolecules in low abundance can form condensates [213]. In addition, the bacterial DNA-binding protein ParB forms nanometer-sized condensates, although these condensates are noncanonical because a motor ParA ATPase regulates the fusion of ParB condensates independently of canonical thermodynamics [214]. Therefore, there can be a number of biomolecular condensates in the steady state even though conventional microscopy currently fails to detect them. We propose that biomolecular condensates are inherent cell volume sensors that recognize bidirectional cell volume perturbation via phase separation/transition to transduce the recognized signal to downstream molecules involved in the subsequent cellular responses.

LLPS potential in cellular volume-changing events

Beyond the primary cell volume sensors, biomolecular condensates may be involved in other cellular volume-changing events. Similar to conditions under hyperosmotic stress, AVD is accompanied by the three aforementioned intracellular changes. Apoptosis is mainly divided into intrinsic and extrinsic pathways, both of which converge at the point of caspase activation [215]. In the intrinsic pathway, the proapoptotic signal induces the release of cytochrome c from mitochondria into the cytosol, and this released cytochrome c subsequently forms a large protein complex called an apoptosome with apoptotic protease activating factor 1 (APAF1) and inactive procaspase-9 [216]. The formation of apoptosomes results in the cleavage and activation of caspase-9, which consequently cleaves and activates caspase-3 and caspase-7. In the extrinsic pathway, apoptosis is initiated by the binding of ligands to death receptors on the cell membrane, including Fas receptors and tumor necrosis factor receptors (TNFRs), followed by the clustering of death receptors and the recruitment of Fas-associated death domain protein (FADD) and caspase-8 [215]. Hence, in both the intrinsic and extrinsic pathways, the clustering of proteins is required for the execution of apoptosis. These clusters are regarded as canonical complexes induced by noncovalent binding between molecules. However, it may be worth reconsidering these processes from the viewpoint of LLPS because AVD potentially satisfies the phase separation/transition requirements. In fact, cytoplasmic adaptor proteins promote the clustering of membrane receptors via phase transition, which provides "signaling zones" [217]. Apoptosis is regulated by multiple signaling pathways; in particular, there is cross talk among the MAPK cascade factors [218]. Thus, phase separation/transition-dependent regulation of apoptosis may be a reasonable way for cells to spatiotemporally control complicated apoptotic/anti-apoptotic signaling pathways.

Cell migration is the repeated cycle of forward protrusion and rear retraction accompanied by cell volume changes. During cell migration, the asymmetrical changes in local osmotic water flow via water and ion-transporting systems and the remodeling of the cytoskeleton play pivotal roles [59, 219, 220]. Recently, the involvement of phase separation in cell migration has been reported; a pair of GTPase regulatory enzymes, GIT1 and β-Pix, formed condensates via phase separation *in vitro* and in cells, and GIT1/β-Pix condensates were proposed to be required for cell migration, probably because they contribute to focal adhesion dynamics via paxillin [221]. The apparent local C_{sat} is dependent on the local microenvironment [213], and the binding of multivalent proteins to the membrane surface induces phase separation at a lower concentration than that required in solution [217]. Therefore, in addition to GIT1/β-Pix condensates, cells may utilize various biomolecular condensates containing water and ion-transporting molecules during cell migration by altering the apparent local C_{sat}. In addition, when the activity and distribution of water and ion-transporting systems are not symmetric within a cell, a gradient of ionic strength/concentration is formed in the cytosol [59], which can trigger phase separation/transition. Hence, biomolecular condensates may maintain sustainable cell migration after the asymmetric regulation of water and ion-transporting systems has been established.

Endocytosis is a trafficking process through which extracellular substances are internalized into the cell, and it is basically accompanied by local cell volume changes. Although still in preprint, a study has proposed that the process of clathrin-mediated endocytosis (CME) membrane invagination is induced by the condensate formation of cytosolic CME proteins with prion-like domains [222]. The adhesion energy produced at the endocytic condensate–cytosol interface can overcome the energy lost during the deformation of the cell membrane and cytosol, implying that endocytic condensates convert the excess free energy derived from phase separation to mechanical forces that induce cell membrane deformation. Hence, cells may leverage biomolecular condensates into effector machinery to generate mechanical forces for cell volume alteration.

Discussion

Cells sense cell volume perturbation through three intracellular changes: 1) change in mechanical forces on membrane proteins, 2) change in intracellular ionic strength/concentration and 3) change in macromolecular crowding. These triad induce the functional alterations of biomolecules, leading to effective cellular responses to cell volume perturbation. Although these functional alterations have been interpreted mainly from the perspectives of biochemistry and molecular biology, we introduced and discussed these intracellular changes from the paradigm of biomolecular LLPS (Fig. 1). Although LLPS and the triad influence each other, they are different dimensions of notion and the perspective of LLPS explains specific features. For example, while the term macromolecular crowding is basically used for the effects unrelated to macromolecular species [136], the property of biomolecular condensates depends on the characteristics of composite biomolecules; hence, the perspective of LLPS covers a regulatory mechanism for a specific biomolecule. Because of ubiquitous water molecules, cells are constantly exposed to stochastic fluctuations in cell volume, and even minuscule cell volume perturbations affect numerous biomolecules and generate "noise" in the cell. Phase separation is induced by delicate changes in macromolecular crowding [159]. Biomolecular condensates can concentrate specific enzymes and substrates to alter their reaction rate and equilibrium specifically [223, 224]. LLPS can reduce the fluctuations of protein concentration in a cell [225]. Therefore, LLPS-induced biomolecular condensates reasonably amplify the "signal" and reduce "noise" (that is, they improve the "signal-to-noise ratio") even during small cell volume perturbations. Furthermore, numerous signaling molecules affect cell volume effectors after cell volume perturbation, but how cells manage to control these molecules simultaneously remains unknown. The perspective of LLPS would provide us with a systematic and generalized understanding of cell volume regulation

with numerous signaling molecules. Until recently, methodology to suggest the direct causal-relationships between biomolecular condensates and physiological events has been limited. For instance, mutagenesis has been utilized to demonstrate the association between malfunction of a mutant in condensate characteristics and the phenomenon of interest, while it potentially generates confounding factors. However, the techniques that control the formation and properties of biomolecular condensates are gradually developed [226], which would facilitate our full assessments of the contribution of LLPS to cellular volume sensing and adaptation. In conclusion, the "lens" of LLPS would enable us to understand the complicated cell volume regulatory mechanism and break through gaps between cell biology and biochemistry/molecular biology interpretations.

Abbreviations

ABC transporter (ATP-binding cassette transporter); AE (anion exchanger); APAF1 (apoptotic protease activating factor 1); AQP (aquaporin); ASK3 (apoptosis signal-regulating kinase 3); ATP (adenosine triphosphate); AVD (apoptotic volume decease); CAPRIN1 (cell cycle associated protein 1); CCC (cation–chloride cotransporter); CME (clathrin-mediated endocytosis); CPSF6 (cleavage and polyadenylation-specific factor 6); DCP1A (decapping mRNA 1A); ECM (extracellular matrix); ENaC (epithelial Na^+ channel); ERK (extracellular signal-regulated kinase); FADD (Fas-associated death domain protein); FMRP (fragile X mental retardation protein); $G\alpha_q$ (G_q protein α subunit); HICC (hypertonicity-induced cation channel); HP1A (heterochromatin protein 1a); K48Ub (K48-linked polyubiquitin); KCC (K^+-Cl^- cotransporter); LATS1/2 (large tumor suppressor 1/2); LLPS (liquid–liquid phase separation); LRRC8A (leucine-rich repeat-containing protein 8A); MAPK (mitogen-activated protein kinase); MEKK2 (MAPK/ERK kinase 2); NCC (sodium–chloride cotransporter); NHE (Na^+/H^+ exchanger); NKCC (Na^+-K^+-Cl^- cotransporter); NLK (nemo-like kinase); OSR1 (oxidative stress-responsive kinase 1); PB (processing body); PEG (polyethylene glycol); PIP (phosphatidylinositol monophosphate); PIP_2 (phosphatidylinositol 4,5-bisphosphate); PKC (protein kinase C); PLC (phospholipase C); PP6 (protein phosphatase 6); PQC (protein quality control); RNP (RNA-binding protein); RVD (regulatory volume decrease); RVI (regulatory volume increase); SG (stress granule); SPAK (STE20/SPS1-related proline/alanine-rich kinase); TAZ (transcriptional coactivator with PDZ-binding motif); TNFR (tumor necrosis factor receptor); TORC1 (target of rapamycin complex 1); TRP (transient receptor potential); VRAC (volume-regulated anion channel); WNK (with-no-lysine [K]); YAP (yes-associated protein).

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Author Contributions

K.W. and H.I. conceptualized and supervised this work. S.I. illustrated the figure and summarized reports into the table. S.I., K.W. and H.I. wrote the manuscript.

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Statement of Ethics
The authors have no ethical conflicts to disclose.

Disclosure Statement

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