REVIEW ARTICLE

Phase separation as a therapeutic target in tight junction-associated human diseases

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Tight junctions (TJs) play an important role in the maintenance of epithelial and endothelial barriers. Zonula occludens (ZO) proteins are scaffolding molecules essential for the formation of TJ complexes, and abnormalities in ZO proteins have been implicated in various TJ-associated human diseases such as tumor invasion and metastasis, and barrier dysfunction. Recent studies reveal that liquid–liquid phase separation of ZO proteins drives the polymerization of TJ proteins into a continuous belt, which then recruits various proteins to form the TJ complex to regulate selective paracellular permeability and signal transduction. Herein, we describe recent advances on how ZO phase separation contributes to TJ formation and discuss the potential of phase separation as a target for the treatment of TJ-associated diseases.

Keywords: tight junction; Zonula occludens; protein–protein interaction; phase separation; paracellular permeability; tumor invasion and metastasis; hypertension; cystic fibrosis; hypomagnesaemia

INTRODUCTION

Tight junctions (TJs) play important roles in the formation of epithelial and endothelial diffusion barriers [1–3]. The integrated TJ complex is composed of several transmembrane and cytoplasmic proteins, including zonula occludens (ZO) proteins. ZO proteins are scaffolding molecules that ensure the formation of an integrated TJ complex that selectively regulates cellular permeability and signal transduction [1, 4]. Several TJ-associated human diseases have been linked to mutations and/or the dysregulation of ZO proteins. The impairment of TJ complexes results in abnormal transport of macromolecules and the disappearance of cell polarity [5, 6]. ZO proteins are also targeted by various pathogens, including pathogenic bacteria and viruses. Cellular components, such as proteins and other macromolecules, are segregated through phase separation to perform specific functions. Phase separation also concentrates molecules into liquid droplets, which facilitates biochemical reactions; the dysregulation of this process can lead to various diseases [7, 8]. In this review, we describe how the phase separation of ZO proteins drives the formation of TJ complexes to regulate paracellular permeability [9, 10]. We also discuss the potential of targeting ZO proteins, with a focus on strategies that modulate phase separation, for the treatment of TJ-associated diseases.

TJ STRUCTURE AND FUNCTION

Epithelial and endothelial cells are connected to adjacent cells through cell–cell junctions located at the most apical part of the lateral plasma membrane. TJs, adherens junctions, and desmosomes are the three major types of junctions [11]. TJs are associated with adherens junctions at the apical plasma membrane and comprise both transmembrane and cytoplasmic proteins [1, 2]. The former integrates adjacent cells, whereas the latter form a dense plaque linking membrane proteins to actin filaments and microtubules. Occludin, claudins, and junctional adhesion molecules are typical transmembrane proteins that mediate cell–cell adhesion. Cytoplasmic plaque proteins include adapter, scaffolding, and cytoskeletal proteins, such as cingulin and membrane-associated guanylate kinase (MAGUK) family proteins. ZO-1 is a typical MAGUK family protein that primarily functions as a scaffold at specific locations within cells [12]. Cingulin is located on the cytoplasmic side of epithelial TJs and interacts with ZO-1, ZO-2, and ZO-3; junctional adhesion molecules; and actin filaments [13]. These various types of TJ proteins interact as part of a complex structural and signaling network [13, 14].

The primary physiological role of TJs is to establish a diffusion barrier that restricts paracellular permeability across the epithelium and endothelium. Permeability to specific ions and macromolecules is determined by their size and charge under a given condition. The mechanism is related to the level of TJ proteins [1, 6]. For example, high expression of occludin promotes the diffusion of small hydrophilic molecules across cultured epithelial monolayers. An intramembrane diffusion barrier establishes cell-surface polarity by restricting the flow of lipids and proteins between the apical and basolateral domains of the plasma membrane; the maintenance of apicobasal cell polarity is necessary for normal cellular activities. The TJ complex recruits various regulatory molecules associated with multiple signaling pathways. For example, ZO-1-associated nucleic acid-binding

protein (ZONAB) is a transcription factor with high affinity for the Src homology 3 (SH3) domain of ZO-1 that participates in cell proliferation; ZO-1 and ZONAB regulate gene expression in a cell-density-dependent manner [15]. In Madin–Darby canine kidney cell monolayers, cell proliferation and density are decreased by the overexpression of ZO-1 and the inhibition of ZONAB, which reduce cyclin D1-mediated G1-to-S–phase transition and decrease the level of proliferating cell nuclear antigen (PCNA) [16].

PHASE SEPARATION AND TJ FORMATION

ZO-1, a 220-kDa peripheral membrane protein, was the first identified TJ protein and has two homologs—namely, ZO-2 and ZO-3. ZO-1 interacts with other ZO proteins and TJ proteins, as well as with the cytoskeleton, through multiple domains to form the TJ complex [17]. ZO-1 has three N-terminal postsynaptic density 95 (PSD95)/Dlg/ZO-1 (PDZ) domains and a carboxy terminus that interacts with actin filaments. ZO proteins (especially ZO-1) contain long intrinsically disordered regions (IDRs) in the central region of the protein that make phase separation possible (Fig. 1a). The dysregulation of proteins involved in phase separation results in the formation of pathological aggregates; for example, the aggregation of tau protein into tangles is observed in patients with Alzheimer's disease [7].

The detailed molecular mechanisms of how TJs are formed are only now starting to be understood. The disruption of ZO-1 results in TJ abnormalities in epithelial cells, highlighting its essential role in TJ formation. The domain organization of ZO proteins facilitates the formation of homo- and heterodimers [18–20]. The conserved PSG (PDZ/SH3/guanylate kinase-like [GUK]) domain induces the polymerization of claudins [17, 21]. It has been proposed that ZO proteins change into condensed scaffolds via phase separation and then drive claudin polymerization and the formation of a continuous TJ belt (Fig. 1b). Live-cell photobleaching experiments demonstrate that ZO proteins are highly dynamic, in accordance with the liquid–liquid phase separation of scaffolding proteins [22, 23]. ZO proteins at high concentrations exhibit liquid-like properties in vivo and in vitro. The concentrations required for spontaneous formation of ZO proteins are significantly higher than the endogenous levels required for their activation in vivo. Both in vitro and in vivo assays have indicated that the PSG supradomain plays a critical role in driving the phase separation of ZO-1 via protein–protein interactions (Fig. 1a) [9].

Membrane-bound ZO proteins facilitate the recruitment and enrichment of other TJ proteins (claudin-1 and occludin), cytoskeletal adapters (afadin and cingulin), and regulators (ZONAB and yes-associated protein), leading to the formation of the TJ complex (Fig. 1b). The phosphorylation state of ZO-1 influences the formation of TJ complexes, with dephosphorylation triggering its phase separation [9, 18, 24]. In a study of gastrulation in zebrafish embryos, the phase separation of ZO-1 is required for TJ formation between the enveloping cell and the yolk syncytial layers. The formation of ZO-1 condensates and their movement toward TJs are triggered by actomyosin tension. Impaired phase separation of ZO-1 results in the loss of TJ mechanosensitivity [10].

PHASE SEPARATION AS A POTENTIAL TARGET

ZO-1 phase separation is essential for TJ complex formation and the maintenance of apical-basal polarity and for ensuring normal paracellular permeability across epithelial and endothelial tissues [25]. Various hereditary TJ-associated human diseases are caused by mutation and the dysregulation of ZO-1, resulting in the loss of cell polarity or compromised barrier function. Decreased levels of ZO-1 and E-cadherin are observed in tumor cell invasion and metastasis, and ZO-1 and ZO-2 are dysregulated along with ZO-1/ZONAB signaling in different types of invasive tumors [1, 2]. In addition, defects in paracellular permeability and associated changes in ion transport are linked to hypertension, cystic fibrosis, and hypomagnesemia. Clarifying the mechanisms underlying the disruption of TJ complex formation and maintenance can provide a basis for developing therapeutic strategies to treat TJ-associated diseases [1, 26].

Tumor invasiveness and metastasis

In human pancreatic cancer, high levels of Zrt- and Irt-related protein 4 (ZIP4) suppress ZO-1 and claudin-1 through interaction with zinc finger E-box–binding homeobox 1 (ZEB1) [27]. The downregulation of ZO-1 promotes tumor growth through focal adhesion kinase (FAK) and paxillin. Based on these observations, inhibiting the ZIP4/ZEB1/ZO-1/claudin-1 pathway is a potential therapeutic strategy in the treatment of pancreatic cancer [28]. Low expression of ZO-1 has been linked to poor prognosis in breast cancer [29]. Several miRNAs have been identified to regulate TJs by targeting ZO-1. For example, miR-25-3p, a cancer-derived exosomal miRNA, participates in the regulation of vascular endothelial growth factor (VEGF) receptor 1 and various TJ proteins through Krüppel-like factor 2 (KLF2). In colorectal cancer, increased miR-25-3 expression results in increased permeability and enhanced metastasis. miR-103 is upregulated in endometrial carcinoma tissues and promotes cell proliferation by directly repressing ZO-1 [30]. The expression of miR-130a is reduced in triple-negative breast cancers (TNBCs), and inhibiting miR-130a reduces the level of ZO-1. High levels of miR-130a inhibit cell migration and invasion through the upregulation of ZO-1 in TNBCs. The effect of miR-130a on ZO-1 can be considered a potential target to suppress cell proliferation in the treatment of TNBCs [31, 32]. Antisense oligonucleotides (ASOs) that mimic the mechanism of action of miRNAs can be used to specifically knockdown repressors of ZO-1 or disrupt its interaction with other molecules to maintain appropriate ZO-1 levels for
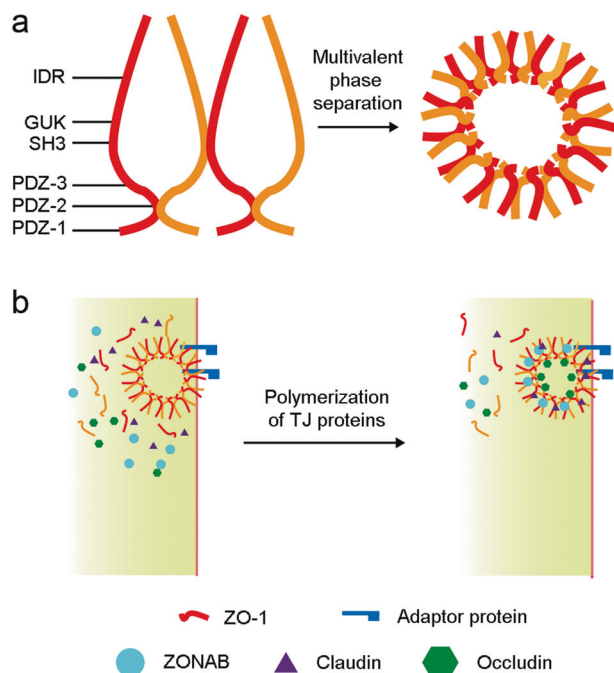

a

IDR
GUK
SH3
PDZ-3
PDZ-2
PDZ-1

Multivalent phase separation

b

Polymerization of TJ proteins

ZO-1   Adaptor protein
ZONAB   Claudin   Occludin

Fig. 1   Model of ZO-1 phase separation driving TJ formation. a The PDZ/SH3/GUK domains and IDRs are essential for the phase separation of ZO-1. **b** ZO-1 first binds to adapter proteins of adherens junctions at the cell membrane. When the threshold concentration is reached, ZO-1 condensates recruit other TJ proteins, such as claudin, occludin, and ZONAB, leading to the formation of a TJ belt.
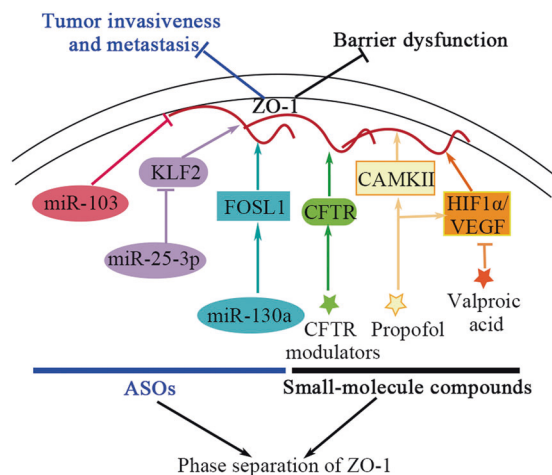
Fig. 2 Mechanisms and strategies of targeting ZO-1 phase separation. A potential therapeutic strategy for the treatment of TJ-associated diseases is the regulation of ZO-1 phase separation by ASOs based on the known actions of miRNAs. Another strategy is based on small-molecule compounds that target ZO-1 to influence its phase separation.

phase separation and TJ complex formation [22]. Taken together, the current evidence suggests that ZO-1 can serve as a prognostic biomarker in cancer and that therapeutic targeting of ZO-1 is a promising strategy for the treatment of cancer and other diseases (Fig. 2).

Barrier dysfunction
The barrier function of TJs is regulated by different mechanisms in various tissues. Cystic fibrosis is induced by the dysfunction of cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-activated chloride channel located in the apical membrane of the secretory epithelium of airways, the intestine, bile ducts, and the epididymis [33, 34]. The PDZ domain of CFTR interacts with ZO-1 to regulate cell proliferation and differentiation via the ZO-1/ZONAB pathway. Inhibiting CFTR reduces ZO-1 expression and the recruitment of ZONAB to TJs, resulting in abnormal proliferation and differentiation [35]. A novel therapeutic strategy for the treatment of cystic fibrosis involves increasing CFTR expression at the cell surface to restore chloride flux [33]. CFTR modulators, such as ivacaftor, lumacaftor, and tezacaftor, have been shown to improve CFTR function and demonstrated clinical efficacy in patients with CFTR Phe508 deletion [36, 37]. The loss of blood–brain barrier (BBB) integrity is associated with neuroinflammation and neurodegeneration-related diseases, including Alzheimer's disease, as well as with postoperative cognitive dysfunction and stroke [38]. Increased membrane permeability and TJ protein downregulation and redistribution are the major features of BBB breakdown, which can be reversed by increasing the expression of ZO-1 [39]. Propofol has neuroprotective effects, including reversing hypoxia and increasing ZO-1 expression and phosphorylation, via the hypoxia-inducible factor 1α/VEGF and calcium/Ca^{2+}/calmodulin-dependent protein kinase II pathways, respectively [40, 41]. Valproic acid, a histone deacetylase inhibitor, inhibits the degradation of ZO-1 through its interaction with hypoxia-inducible factor 1α (HIF1α)/VEGF signaling, thereby protecting against burn-induced gut barrier dysfunction [42]. Thus, small-molecule compounds indirectly alter paracellular permeability by inducing the expression or degradation of ZO-1 (Fig. 2). High-throughput screening can potentially identify novel small-molecule compounds that modulate ZO-1 phase separation, and these compounds may be effective for treating diseases related to barrier dysfunction.

CONCLUDING REMARKS
Liquid–liquid phase separation is important for the regulation of many dynamic assemblies, including TJ complexes. ZO-1 and PSD95 are MAGUK family proteins that contain multiple protein–protein interaction domains. Phase separation of ZO-1 and PSD95 drives the formation of TJ complexes and the organization of postsynaptic densities in neurons, respectively. ZO-1 condensates not only recruit components of the TJ complex but also facilitate the binding of signaling proteins. These signaling proteins enable the important role of ZO-1 in barrier formation and its physiological roles in cell proliferation and differentiation. Given its widespread cellular and tissue distribution, it is unsurprising that aberrant ZO-1 expression and activity have been implicated in a number of pathophysiological conditions in humans. Deficiency in ZO-1 or other ZOs has been reported in a series of diseases. However, whether excessive expression of ZO-1 is involved in any confined disease is not clear. A high level of ZO-1 might result in abnormal aggregation that influences the recruitment of other TJ proteins or regulators. The level of ZO-1 should be fine-tuned by miRNAs or small-molecule compounds during disease treatment. In this scenario, gradient concentrations of miRNAs or small-molecule compounds should be examined to control ZO-1 to the appropriate level. Moreover, both the method for drug delivery and the duration of drug treatment need to be taken into account for disease treatment.

Determining the conditions that are necessary for the phase separation of ZO-1 may lead to the discovery of an effective treatment for TJ-associated diseases, including cancer. Changes in phosphorylation state, temperature, ion concentration, pH, and the addition of certain drugs have been shown to influence the phase separation of key proteins [26, 43]. The PDZ3-SH3-GUK domain, the core of the MAGUK protein family that participates in various signaling pathways, is unsuitable for the targeting point [21]. However, the IDR of ZO-1 might be targeted to modulate phase separation for its essential role in this process. ASOs that specifically knockdown ZO-1 regulators—which are less widespread than ZO-1 itself and are, therefore, more useful targets—can restore normal levels of ZO-1 and enable its phase separation. Small-molecule compounds, such as propofol and valproic acid, that do not directly target ZO-1 but enhance BBB integrity are another option [40, 42]. The most effective strategy for the identification of such compounds is to screen those that can bind to ZO-1 and have the chemical properties required to influence ZO-1 phase separation. Further investigation is needed to elucidate the molecular basis of TJ-associated diseases so that suitable ASOs can be designed and small-molecule compounds can be identified based on the structure of ZO-1, especially the domains essential for its phase separation.

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