N-terminal tail prolines of Gal-3 mediate its oligomerization/phase separation

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Galectins are a class of proteins that bind to β-galactose–containing glycoconjugates and play critical roles in developmental, homeostatic, and pathological contexts (1, 2). Expressed across animal tissues, galectins are synthesized in the cytoplasm and trafficked to the extracellular milieu via the unconventional secretion pathway, although they may also be localized within the cytoplasm and nuclei of cells (3). Secreted galectins bind to cell surface glycoconjugates and modulate adhesion of cells to each other and to the extracellular matrix, as well as exert influence on intracellular signaling (4, 5). All galectins possess one or two evolutionarily conserved carbohydrate recognition domains (CRDs) that are responsible for binding their cognate sugars (6). Shaped like a jelly roll, the structure of a CRD consists of two opposing β-sheets: a five-stranded F-face and a six-stranded sugar-binding S-face. Among galectins, only galectin-3 (Gal-3) belongs to the chimera type: It is characterized by a long and intrinsically disordered proline-rich N-terminal tail (NT) (7). Binding to cell surface glycoconjugates induces secreted Gal-3 to oligomerize through mechanisms that are as yet incompletely understood; quantitative precipitation studies indicate that Gal-3 may pentamerize in the presence of multivalent glycan ligands and form heterogeneous disorganized complexes with the latter (8). The multimerization of Gal-3 has been proposed to occur both by self-association through its C-terminal CRDs as well as through self-association of its NTs (9). In addition, the NT has been shown to form intramolecular links with the F-face of Gal-3 CRD (which implies a potential unification of the self-association models) (10). However, the exact role of NT in the specific cellular functions of Gal-3 is still unclear. Equally enigmatic is the presence of multiple prolines in the NT of Gal-3.

In PNAS, Zhao et al. (11) address these important questions. By creating a series of Gal-3 mutants that have their individual prolines mutated to alanines (and in one case to histidine in accordance with a P64H polymorphism that is associated with pathologies such as breast and thyroid cancer), the authors show that the removal of individual NT prolines impairs the ability of Gal-3 to induce human endothelial cell migration, T cell activation, and erythrocyte agglutination [all classically described functions of Gal-3 (12–14)]. The extent of impairment depends on the identity of the mutated proline; moreover, different, if overlapping, sets of prolines seem to contribute to each of the above cellular functions. Gal-3 has earlier been shown to mediate the formation of structures known as clathrin-independent carriers responsible for endocytosis of cell surface proteins such as CD44 and integrins (15). A set of NT prolines (different from the set inducing migration in the same cells) are also found to be necessary for the successful dispensation of this function of Gal-3.

Employing a series of biophysical techniques, the authors show that in the presence of glycoprotein ligands, Gal-3 may aggregate through a phenomenon akin to liquid–liquid phase separation (LLPS). The latter pertains to the sequestration of chemical reactions and the molecules mediating them in a membrane-independent manner through phase separation from the surrounding liquid milieu (16, 17). Zhao et al. show that this aggregation is dependent on the concentrations of Gal-3 and its ligands. By testing droplet formation using covalently delinked Gal-3 CRDs and NTs, they demonstrate that intermolecular interactions between the NTs and F-faces of the CRDs may drive LLPS-like behavior. This separation is impaired by attenuating the binding of the S-face of CRD to the glycoprotein ligands as well as by mutating the prolines of the NT, as described above. To a lesser extent, intermolecular NT–NT interactions also contribute to the aggregation. Taken together, these results suggest a model of galectin-ligand phase separation that is dependent on interactions between NT and CRD F-face within and across Gal-3 proteins, between Gal-3 NTs, and of CRD S-face with cognate glycan moieties of glycoproteins.

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LLPS plays a vital role in the formation of nonorganellar complexes such as stress granules, P bodies, Cajal bodies, nuclear speckles, promyelocytic leukemia (PML) bodies, nucleoli, etc. (17). Such complexes sequester high levels of proteins that can interact and function in a spatially specific manner. Although seen extensively inside cells wherein both DNA and RNA can also participate in phase separation (18, 19), there is sparse evidence for the extracellular occurrence of such condensates. The presence of intrinsically disordered or low-complexity amino acid stretches and the propensity to participate in relatively weak but multivalent interactions are canonical characteristics of proteins that phase-separate within cells. The frequent prevalence of such properties even in proteins outside cells, including Gal-3, suggests that the extracellular microenvironment may well be an exciting, if not easily tractable, hub of LLPS (20–22).

The association between Gal-3 and phase separation is not new. The presence of Gal-3 in nuclear bodies that are now considered exemplars of LLPS was demonstrated in a series of influential papers by Ronald Patterson's group (23, 24). More recently, intramolecular and intermolecular associations between the intrinsically disordered NT and Gal-3 CRD were observed using NMR spectroscopy, small-angle X-ray scattering, and computational modeling, leading to the proposal that hydrophobicity-driven fuzzy interactions drive Gal-3 to self-associate and phase-separate (24, 25). A micellar model of Gal-3–driven LLPS has subsequently been proposed; this model is dependent on the lactose-binding function of Gal-3 CRD and its NT aromatic residues such as tryptophan and tyrosine (25). The aromatic residues of the NT mediate both intramolecular and intermolecular interactions with the F-face of Gal-3 CRD. Zhao et al. go further and demonstrate LLPS-based oligomerization in a cellular context using binding studies with fluorescently labeled and unlabeled Gal-3. Competition assays reveal that Gal-3 molecules enhance each other's binding on cell surfaces in the presence of their glycan ligands. Not just that, at physiologically appropriate levels, CRD F-face–NT interactions predominantly contribute to condensation (more than NT–NT associations that are seen for assays using supraphysiological levels of Gal-3).

How does the mutagenesis of individual prolines of Gal-3 NT lead to distinct consequences on Gal-3–driven cell functions? Zhao et al. partially address this question by showing that the mutations of specific NT prolines alter the profile and avidity of binding of the Gal-3 mutants to an array of N-linked glycans. Intriguingly, the prolines that are closest to the Gal-3 CRD seem to exert a greater influence on the protein's ability to mediate the described cellular functions. The authors show that these prolines are also the ones that contextually determine the binding of the protein to cell surface glycans. Nevertheless, the sizes of droplets formed by individual Gal-3 mutants in the presence of glycoproteins such as CD45, CD146, CD7, and CD71 do not show a pattern with the spatial distance of the mutated proline from the Gal-3 CRD, suggesting that the contributions of NT prolines to droplet formation and cellular function may overlap but are not completely congruent.

The prolific deployment of LLPS to explain the space-specific condensation of molecules within a biological milieu has led to efforts to demonstrate its relevance in vivo (26). On the other hand, aggregation of proteins may also occur through multiple mechanisms and principles that may superficially resemble, but do not involve, liquid phase separation (27). Therefore, it is pertinent to arrive at a stringently applicable set of quantitative criteria to distinguish between phase separation and other routes of achieving locally high concentrations of proteins, as well as test the utility of LLPS as an aggregative mechanism within physiological regimes

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of protein concentrations. Zhao et al. have taken care to design their cellular experiments with Gal-3 concentrations that fall within physiological limits. Looking ahead, it would be fascinating to verify the ability of Gal-3 to phase-separate outside cells within animal and organoid models. Probing the same in cancer, which is characterized by dysregulation of cell-surface glycan expression on the one hand and perturbed galectin levels and localizations on the other (28, 29), will likely add a new layer of regulation to the mechanisms of malignant transformation.

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