Assemblages: Functional units formed by cellular phase separation

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The partitioning of intracellular space beyond membrane-bound organelles can be achieved with collections of proteins that are multivalent or contain low-complexity, intrinsically disordered regions. These proteins can undergo a physical phase change to form functional granules or other entities within the cytoplasm or nucleoplasm that collectively we term "assemblage." Intrinsically disordered proteins (IDPs) play an important role in forming a subset of cellular assemblages by promoting phase separation. Recent work points to an involvement of assemblages in disease states, indicating that intrinsic disorder and phase transitions should be considered in the development of therapeutics.

A remarkably large proportion of proteins in eukaryotic proteomes lack folded globular structure and are intrinsically disordered under physiological conditions (Wright and Dyson, 1999; Dunker and Obradovic, 2001; Tompa, 2002; Dyson and Wright, 2005). Intrinsically disordered proteins (IDPs) or intrinsically disordered regions (IDRs) play a central role in numerous cellular processes and are directly implicated in human diseases that include cancer and neurodegenerative and amyloid diseases. IDPs were originally identified and characterized by biochemical and spectroscopic methods (Kriwacki et al., 1996; Weinreb et al., 1996; Daughdrill et al., 1997), but can be readily identified by sequence analysis because of their biased amino acid composition and, in particular, their low content of hydrophobic residues, which prevents them from folding spontaneously (Romero, P., Z. Obradovic, C. Kissinger, J.E. Villafranca, and A.K. Dunker. 1997. Proceedings of the International Conference on Neural Networks. http://dx.doi.org/10.1109/ICNN.1997.611643; Xie et al., 1998; Romero et al., 2001; Vucetic et al., 2003; Dyson and Wright, 2005). Bioinformatic surveys of entire genomes reveal that disordered proteins are highly abundant in eukaryotes, with ∼40% of proteins in the human proteome containing long disordered regions (Ward et al., 2004; Pentony and Jones, 2010).

The proportion of proteins that contain disordered segments increases with increasing complexity of the organism (Dunker et al., 2002; Ward et al., 2004). Neural proteins and proteins involved in eukaryotic signal transduction or associated with cancer have an even higher propensity for intrinsic disorder; 60% of proteins in a human cancer protein database are predicted to be disordered over 50 or more contiguous residues (Iakoucheva et al., 2002). IDPs act as central hubs in signaling networks; their abundance is tightly regulated to maintain signaling fidelity, and changes in cellular levels are associated with pathologies (Gsponer et al., 2008; Vavouri et al., 2009).

Many IDRs contain short recognition motifs that mediate interactions with their cellular targets (Wright and Dyson, 1999; Dunker et al., 2005; Dyson and Wright, 2005; Mohan et al., 2006). Such motifs are commonly amphipathic and fold into ordered elements of structure upon binding to a target protein (Wright and Dyson, 1999, 2009). Not all IDRs adopt folded structures. Some appear to function as flexible linkers between structured domains (Dyson and Wright, 2005), whereas others remain disordered even when bound to targets (Baker et al., 2007; Mittag et al., 2008, 2010), forming complexes that have been described as "fuzzy" (Tompa and Fuxreiter, 2008).

In performing their regulatory and signaling functions, IDPs tend to make discrete interactions with binding partners, forming complexes with well-defined stoichiometry. However, in recent years, a new function has been recognized for a subset of IDPs that contain low-complexity regions in which many, but not all, of this subset can undergo large-scale association through homotypic or heterotypic multivalent interactions (see van der Lee et al., 2014). These IDPs can undergo phase transitions, leading to separated liquid droplets, hydrogels, and protein aggregates or fibrils (Vekilov, 2010). In this process, a homogenous protein solution separates into a dilute supernatant, and a protein-rich phase formed through an extensive network of weak, multivalent protein–protein interactions. The physical chemistry of phase separation is well understood (Pappu et al., 2008), and the process is dependent upon protein

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Abbreviations used in this paper: ES, Ewing sarcoma; FG, phenylalanine-glycine; IDP, intrinsically disordered protein; IDR, intrinsically disordered region.

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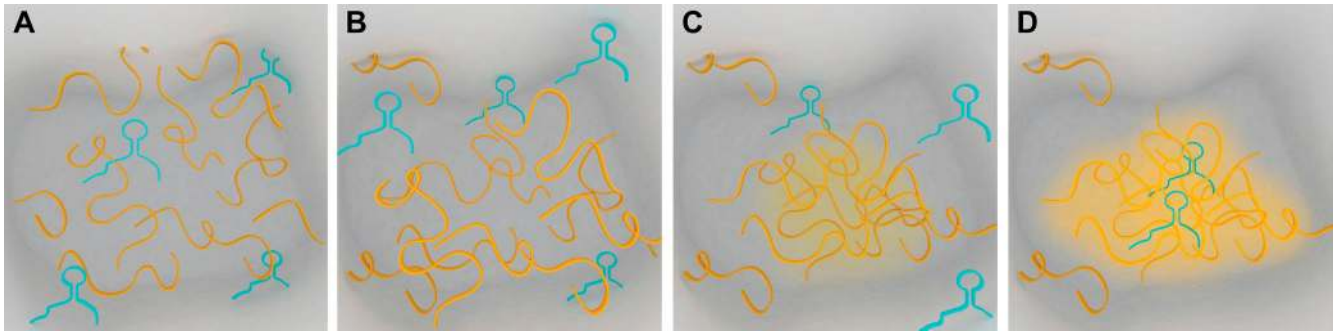

Figure 1. **Assemblage formation leads to emergent properties of protein and RNA binding.** This series of panels (A–D) demonstrates that an increase in the local concentration of protein (yellow ribbons) in regions of a cell can result in a phase transition (yellow haze) to form an assemblage once a critical concentration has been reached. A phase separated assemblage can be formed through weak homotypic or heterotypic interactions and allows exchange of constituent molecules with the surrounding solution. This phase-separated material allows for the capture and interaction of other protein or RNA species (cyan molecule). (D) The final assemblage formation shows the sequestration of two RNA molecules.

concentration, the degree of multivalency, and the strength of the intermolecular interactions. Protein phase transitions have recently received much attention because of a growing body of evidence that phase separation plays a functional role in the microscopic organization of the cell (Weber and Brangwynne, 2012; Kedersha et al., 2013; Tompa, 2013). These processes, their relationship to intrinsic protein disorder, and their connection to disease form the focus of this review.

IDPs promote phase separation to create intracellular partitions

Many cellular functions are performed within organelles that are enclosed within lipid membranes. However, other functions depend upon assemblies of proteins and nucleic acids that are not membrane bound. Through a process of phase separation, biological macromolecules can form distinct compartments in either the cytoplasm or nucleoplasm. These assemblies were first observed in cells as granules, but hardly distinguishable from metabolic granules, such as the lysosome, by electron microscopy (Novikoff, 1956). The functional compartmentalization of intracellular space can be considered parallel to lipid rafts that cause coalescence of transmembrane receptor proteins. André and Rouiller (1957) identified and described dense material that lacked a membrane, often perinuclear or accompanied by mitochondria in *Drosophila melanogaster* germ cells, which they termed "nuage." The term nuage, meaning "cloud" in French, has been used to describe not only the cytoplasmic regions of germ cells in *Drosophila*, but is also being extended to other organisms based upon investigation of homologous proteins.

There are many macromolecular assemblies that have been identified in either the cytoplasm or nucleus, including cytoplasmic P granules, germ cell granules, and various nuclear bodies (nucleoli, Cajal bodies, PML bodies, speckles, etc.), but these particles lack a clear unifying terminology. Thus, we propose the unifying name of "assemblage" for all of these functional particles that involve phase transitions, some of which are made up at least in part from IDPs. These particles exhibit liquid-like behavior, and their components are in constant and rapid exchange with the surrounding cytoplasm or nucleoplasm (Misteli, 2001; Brangwynne et al., 2009). A key property of assemblages formed by repetitive, low-complexity, IDP sequences is reversibility.

Protein–protein or protein–RNA interactions in an assemblage are multivalent and dynamic, and can be mediated by IDRs of low-complexity or multivalent folded protein domains (Fig. 1). IDPs play an important role in assemblages based upon their ability to dynamically associate either homotypically or heterotypically. The emergent properties that occur in response to a phase transition include the ability to bind RNA or protein in novel interactions (Fig. 1). Polymer physics, including the theories of P. Flory and W. Stockmayer, can contribute to the numerical modeling of assemblages (for a review and detailed discussion of analytical models, see Falkenberg et al., 2013). To discuss the phenomenon of assemblage, an understanding of the biochemical and biophysical underpinnings of phase transitions leading to separation is necessary.

Liquid–liquid demixing can lead to a type of phase transition, where microscopic liquid droplets that are rich in proteins or RNA separate from the cytoplasm or nucleoplasm (Fig. 1). The vast majority of demixing studies to date have involved in vitro systems, but in vivo evidence is growing. These phase transitions are driven by intermolecular interactions and are strongly dependent on the concentration of the protein and/or RNA molecules that partition into the liquid droplets. Early studies that identified the macromolecular interactions that promote phase separation used synthetic elastin-like polypeptides (Martino et al., 2000; Meyer et al., 2001). In addition, microstate partitioning models were developed with peptides such as polyglutamine, where as few as 15 glutamine residues cause the peptide to form a collapsed state with poor aqueous solubility (Pappu et al., 2008). Glutamine, as a hydrophilic amino acid, has excellent water solubility as a monomer, but polyglutamine develops significantly different properties based upon the number of glutamine residues, the concentration, and the chemical environment. This relatively simple model allows the nonbiophysicist to begin to visualize how phase transitions occur as the composition of the polypeptide changes or its concentration increases. The critical concentration required for a change in phase of a protein depends upon its sequence and its environment; this is a key starting point for discussing the concept of phase transition

leading to particle formation by phase separation. Advances in modeling of IDPs based upon the amino acid sequence will inform future understanding of in vivo assemblage formation and the contribution of IDPs to this process (Mao et al., 2010; Müller-Späth et al., 2010; Das and Pappu, 2013; Soranno et al., 2014).

Although intrinsic protein disorder plays an important role in formation of cellular assemblages, these can also be formed by interactions between folded proteins that promote phase transitions. Phase separations can occur when the interacting macromolecules reach a critical concentration and are aided by multivalency (Fromm et al., 2014). One model of assemblage formation was nicely demonstrated using engineered proteins containing 1–5 SH3 domains or 1–5 proline-rich motifs (PRMs; ligands for SH3 domains) that were mixed combinatorially to study the effects of concentration and valency on phase separation (Li et al., 2012). A valency of 4 significantly lowers the threshold concentration, leading to a cooperative phase transition for PRM binding to SH3 domains (Li et al., 2012). A naturally occurring multiprotein system consisting of nephrin, NCK, and N-WASP confirmed the importance of multivalency by showing that tyrosine phosphorylation increased the valency of the NCK SH2 domain binding to nephrin. This led to a local increase in the concentration of the SH3 domains of NCK, leading to N-WASP binding as the phase transition occurs. When the solution transitioned to gel phase, there was a large increase in the rate of Arp2/3-mediated actin assembly. This phase transition, however, occurred independently of Arp2/3 binding (Li et al., 2012).

Low-complexity IDPs, b-isox, and hydrogels

A biotinylated version of 5-aryl-isoxazole-3-carboxylamide (Sadek et al., 2008) with a saturated linker, now called b-isox, was serendipitously shown to reversibly precipitate proteins with low-complexity sequences, including FUS, EWS, TIA-1, ATXN2, FXR1, and other RNA-binding proteins (Kato et al., 2012). Extensive studies of FUS showed that a low-complexity region of the sequence, containing 27 [G/S]Y[G/S] repeats, mediates a concentration-dependent phase transition to a hydrogel state. This hydrogel was capable of retaining FUS and other RNA-binding proteins, a property that was lost when the critical tyrosine residue was mutated to serine. Biophysical studies revealed that the hydrogel state is composed of polymerized amyloid-like fibers (Kato et al., 2012). The authors hypothesized that the low-complexity sequences play a functional role in controlling exchange of proteins into and out of subcellular granules through reversible formation of dynamic amyloid fibers. It is of note that the protein components of RNA granules are highly enriched in low-complexity [G/S]Y[G/S] sequences (Kato et al., 2012).

B-isox forms a crystal lattice that has long narrow pores that appear to entrap low-complexity sequences based upon their formation of fibers (Kato et al., 2012). These fibers are also responsible for the trapping of specific RNA species in the hydrogel. A highly significant correlation exists between those mRNAs that are trapped in hydrogels compared with those precipitated by b-isox (Han et al., 2012). The mRNA molecules that are found in the hydrogels of FUS and EWS appear relatively distinct and suggest a level of "specificity" based upon a comparison of trapped mRNA identified from reduction of either FUS or EWS, or of both proteins (Han et al., 2012). DNA-dependent protein kinase phosphorylation of serine leads to release of mRNA from the FUS hydrogel, which suggests that granule disassembly may be controlled by posttranslational modification (Han et al., 2012). These findings provide a framework for further investigations to identify the mechanistic and structural rationale for specificity between RNA sequences and protein binding that occurs in assemblage.

Cytoplasmic bodies exhibit phase transitions

The cytoplasm contains RNP assemblages whose existence and function depend upon localized phase transitions that lead to phase separation. Intrinsically disordered, low-complexity amino acid sequences are critical to the phase transition of the PGL family of proteins that function in assembly of cytoplasmic RNP granules (Brangwynne et al., 2009; Updike et al., 2011). Germline P granules, which are a type of RNP granule, are critical for the polarization that leads to asymmetric cell division of the one-cell embryo (for review see Gao and Arkov, 2013). Phase separation accounts for the development of cell polarity by posterior localization of P granules. The P granules have the physical properties of liquid droplets and are in equilibrium with their soluble components (Brangwynne et al., 2009). The granules do not move as an intact assemblage through the cytoplasm. Rather, movement occurs by rapid dissolution and condensation of the granule (Brangwynne et al., 2009). Individual proteins that comprise the P granule do diffuse through the cytoplasm. After symmetry breaking, concentration gradients of the polarity proteins MEX-5 and PAR-1 promote posterior localization of the granule. MEX-5 promotes granule dissolution, whereas PAR-1 antagonizes MEX-5 and effectively lowers the concentration required for phase separation of granule components through weak homotypic or heterotypic interactions. This dynamic process of local assembly and dissolution leads to the localization of P granules in one pole of the embryo before mitosis (Brangwynne, 2013), thereby establishing polarity.

Germ cell granules in amphibians are nucleated by the IDP Xvelo1 (Nijjar and Woodland, 2013) and in vertebrates by bucky ball, which contains IDRs (Marlow and Mullins, 2008; Bontems et al., 2009). Mammalian oocytes do not contain germ cell granules per se, and do not use the same mechanisms to establish polarity. However, many of the homologous proteins that compose germ cell granules, such as *TNRC6A* (also known as *GW182*), which is an IDP, are present in primordial mammalian cells (Voronina et al., 2011). In HeLa cells, P granule (also known as stress granule) formation is caused by DYRK3 activation of mTORC1, in part through phosphorylation of the IDP PRAS40 (Wippich et al., 2013).

The concentration-dependent phase transitions of the assemblages result in liquid droplets with a significantly higher density than the surrounding liquid cytoplasm or nucleoplasm. Some investigators refer to these higher-density liquids in cells

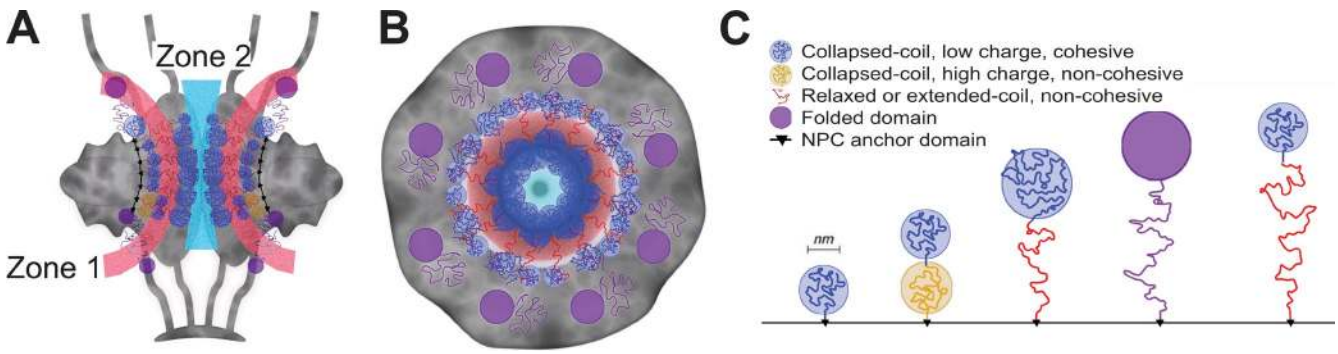

Figure 2. **Phase transition caused by IDPs regulates transport in nuclear pore channels.** (A) Sagittal plane of a nuclear pore that is populated with IDPs. FG proteins, and variants described in the text, contribute to the "filling" of space between structural protein components (gray). The architecture of the NPC creates zones of transport, indicated here as light red and light blue. (B) Transverse view of nuclear pore complex components (Nups) showing the localization of FG proteins and the transport zones. (C) Legend demonstrating the types of proteins found in the nuclear pore complex and their biophysical characteristics. This figure is based upon *Saccharomyces cerevisiae* FG Nups and is adapted from Yamada et al. (2010).

as hydrogels, based upon their physical appearance and properties in vitro (Han et al., 2012). As described earlier, these hydrogels can "intercept" other P granule components, such as the germ line helicase GLH-1 (Brangwynne et al., 2009). The size exclusion "barrier," or filter, of the P granule is formed by incorporation of GLH proteins, which contain phenylalanine-glycine (FG) low-complexity repeats reminiscent of the nuclear pore complex (Updike et al., 2011). The protein PGL-1, which contains low-complexity GYG repeat motifs, is critical for the GLH condensation and localization to the nuclear pore. The P granule barrier excludes proteins larger than 45 kD, thereby acting as a nuclear transport filter (Updike et al., 2011). Thus, when a P granule associates with the nuclear pore complex, a size filter to the nuclear transport of macromolecules can occur (Updike et al., 2011).

The nuclear pore complex: putative function of phase separation in transport

Although the shell of the nuclear pore is an ordered structure, the cytoplasmic surface of nuclear pores is surrounded by proteins, such as Nup159, with low-complexity FG sequences that form fibrillar extensions into the cytoplasm and act as a primary barrier for large molecular species (Patel et al., 2007). One of the critical functions of the nuclear pore is the regulated transport of proteins into the nucleus, but how this occurs remains cryptic, despite multiple transport models (Rabut and Ellenberg, 2001; Adams and Wente, 2013). Current understanding of nuclear pores describe three main types of proteins: transmembrane Nups that anchor the nuclear pore complex in the nuclear envelope, structural Nups that stabilize the nuclear envelope curvature and provide scaffolding, and intrinsically disordered FG Nups that contribute to the permeability barrier for nonspecific transport and facilitate movement as direct binding sites for transport receptors (Adams and Wente, 2013).

Identification of FG repeats as low-complexity secondary sequences in the nuclear pore complex, first in yeast (Wente et al., 1992; Wimmer et al., 1992), then in mammalian cells (Radu et al., 1995), was a critical step in modeling NPC transport. These FG repeats were experimentally validated as intrinsically disordered, and hypotheses concerning their roles in transport have evolved (Denning et al., 2003).

Although FG repeats are a broad class of Nup, sequence variations such as FxFG and FLFG, along with FG, are differentially distributed at the cytoplasmic, intra-NPC, or nucleoplasmic regions of the NPC (Atkinson et al., 2013). A mutagenesis analysis of the role of FG repeat Nups in yeast suggests that multiple pathways exist for transport through the nuclear pore (Strawn et al., 2004). The FG Nups are structurally heterogeneous, adopting a spectrum of disordered conformational states that range from collapsed coils to highly extended configurations (Yamada et al., 2010), leading to a zonal model of the nuclear pore (Fig. 2). Additional investigations support a critical role for FG nucleoporins in the transport of mRNA, including relative specificities for GLFG and FxFG repeat sequences (Terry and Wente, 2007).

Karyopherins, a class of nuclear transport proteins from multiple species from yeast to human, have been found to bind to FG repeats. The nature of the interactions of FG repeats with transporter proteins is highly dependent on their hydrophobic microenvironments; cells show diminished growth when FG repeats are disrupted by mutagenesis or by aliphatic alcohols (Patel et al., 2007). In vitro physical models of FG repeat proteins demonstrate that phase transition occurs from liquid to hydrogel, in support of a phase separation model for the core of the nuclear pore (Frey and Görlich, 2007; Ader et al., 2010; Diesinger and Heermann, 2010). A detailed evaluation of nearly all Nups and their contribution to transport supports phase transition leading to assemblage formation in the nuclear pore complex transport system (Yamada et al., 2010).

Model NPCs have been reconstituted from *Xenopus laevis* egg extracts under near in vivo conditions, allowing direct measurements of the permeability over time (Hülsmann et al., 2012). These experiments suggest a hydrogel-like phase transition that regulates the nuclear pore barrier based upon the local concentration of low-complexity residues in the NPC. In contrast, polarized fluorescence microscopy suggests a more structurally ordered environment for NPCs within the cell, described as nematic ordering, in which molecules have no positional order but are self-aligned to have long-range directional order, with their long axes roughly parallel (Atkinson et al., 2013). Using high-resolution EM imaging, a dynamic picture of GLFG

repeats shows movement in a concentration gradient–dependent fashion, but only in specific regions (Fiserova et al., 2014). In summary, these different paradigms of nuclear transport indicate that IDP composition leading to phase transition is crucial for this essential cellular process (Adams and Wente, 2013).

Phase separation as a mechanism for protein interaction in disease

Biophysical studies have clearly documented phase transitions that lead to phase separation of proteins in vitro, whereas cellular studies demonstrate that phase separation with creation of assemblage occurs as part of critical biological processes in vivo. For example, a phase transition plays a central role in assembly of the myelin sheath, driving association of the intrinsically disordered myelin basic protein to form a mesh-like network on the inner leaflet of the membrane bilayer (Aggarwal et al., 2013). The low-complexity amino acid motifs that cause phase separation may also drive pathology. Certain mutant proteins considered to be etiologic in neurodegenerative diseases and cancer have emergent properties that suggest that phase separation leads to aggregation. The fibrillar aggregates in neurodegenerative disease are not considered reversible from a pathological standpoint, or at least have not been shown to be at this time. The lack of reversibility in these terminal aggregates therefore does not fully fit within our definition of assemblage. We discuss these aggregates, however, because they clearly derive from IDPs and their formation is driven by phase transitions.

Huntington's disease is a neurodegenerative disorder caused by polyglutamine repeat expansions in the huntingtin protein (Lee et al., 2014). The expansion of polyglutamine in mutant huntingtin (mHTT) results in formation of fibrillar aggregates that sequester and inhibit the transcriptional regulatory functions of CREB-binding protein (CBP; Nucifora et al., 2001). mHTT aggregates also sequester and disrupt the functions of additional regulatory proteins, including specificity protein 1 (SP1), TATA box binding protein (TBP), the TFIID subunit TAFII130, the RAP30 subunit of the TFIIF complex, and the CAAT box transcription factor NF-Y (Kim et al., 2002; Labbadia and Morimoto, 2013). To probe the effect of increasing the number of glutamine residues in huntingtin, terminal cyan and yellow fluorescent proteins were introduced into the N17 and polyproline regions that flank the polyglutamine tract (Caron et al., 2013). When the length of the polyglutamine tract exceeded 37 residues, a decrease in Förster resonance energy transfer (FRET) was observed, which indicates conformational changes and disruption of interactions between the N17 and polyproline regions. Polyglutamine tracts have the ability to self-assemble into fibrillar aggregates (Burke et al., 2003). Aggregation of disordered, low-complexity polyglutamine tracts is thought to occur by a process of liquid–liquid demixing, leading to phase separation, and both the kinetics and aggregation propensity are modulated by the flanking regions (Fiumara et al., 2010; Crick et al., 2013).

Many other neuropathological diseases involve a type of assemblage called stress granules, and their role in disease has been recently reviewed (Li et al., 2013). These assemblages condense based upon prion-like sequences (also known as low-complexity sequences) present in mutant TDP-43 (Arai et al., 2006; King et al., 2012), FUS (King et al., 2012), hnRNPA2B1, and hnRNPA1 (Kim et al., 2013). These proteins, whose function involves RNA binding, have IDRs and undergo phase transitions that lead to formation of granular assemblages (Malinovska et al., 2013). Pathogenicity is thought to derive in part through aberrant sequestration of RNA in these aggregates (Gitler and Shorter, 2011).

Mutant p53 is a well-recognized driver of cancer initiation, progression, and maintenance of the cancer phenotype. The central region of wild-type p53 (p53C) can nucleate fibril formation, but this seldom happens under normal physiological conditions (Ishimaru et al., 2003, 2004). Mutant p53, in particular the 30% of mutations that lead to unfolding of the protein, have a greater propensity for spontaneous fibril formation (Xu et al., 2011). These fibrils can occur in the nucleus of cells with mutant p53, as demonstrated in breast carcinoma with R248Q (Ano Bom et al., 2012). Data suggest that p53 fibrils can nucleate assemblage and even pass this nucleated assemblage onto other cells through micropinocytosis (Forget et al., 2013; Hofmann et al., 2013).

Protein phase transitions in disease: the cause and the cure?

Previous reviews have described IDPs as integral to pathogenesis in a wide spectrum of human disease (Midic et al., 2009). The potential reversibility of these pathogenic protein complexes remains unresolved, and probes are needed to test reversibility of the protein aggregates; these probes will both inform on the biochemistry of the interaction as well as provide insights into the contribution of the aggregates to pathogenesis.

The function of the TET (TLS [or FUS]-EWS-TAF15, also known as FET) family of proteins remained relatively obscure for many years after their discovery, in part because of their seemingly promiscuous involvement in biological processes via RNA binding (Lee, 2007). Proteomic analysis has identified the interacting partners of TET proteins, which include proteins from all aspects of transcription and mRNA processing (Pahlich et al., 2009). These TET proteins can participate in or drive pathological processes through three mechanisms. First, chromosomal translocation of EWS in Ewing sarcoma (ES) became recognized as a fusion partner in many cancer phenotypes bearing little phenotypic relationship to ES (Delattre et al., 1992; Kovar, 2011). Second, EWS or EWS-FLI1 can participate in protein interactions, either by formation of defined complexes or putatively as an assemblage. EWS participation in splicing foci thus represents a potential biological example where phase separation is not yet proven, but may play a role in disease (Paronetto et al., 2011). Third, mutations in EWS that lead to protein aggregation in ALS are focusing attention on EWS as an aggregation-prone protein that can nucleate or become involved in aberrant cellular assemblages (Couthouis et al., 2012).

The intrinsically disordered, low-complexity region of EWS, like the other TET proteins, contains repeats of the [G/S]Y[G/S] motif as well as the sequence SYGQQS, a repetitive glutamine-rich motif with prion-like properties (King et al., 2012;

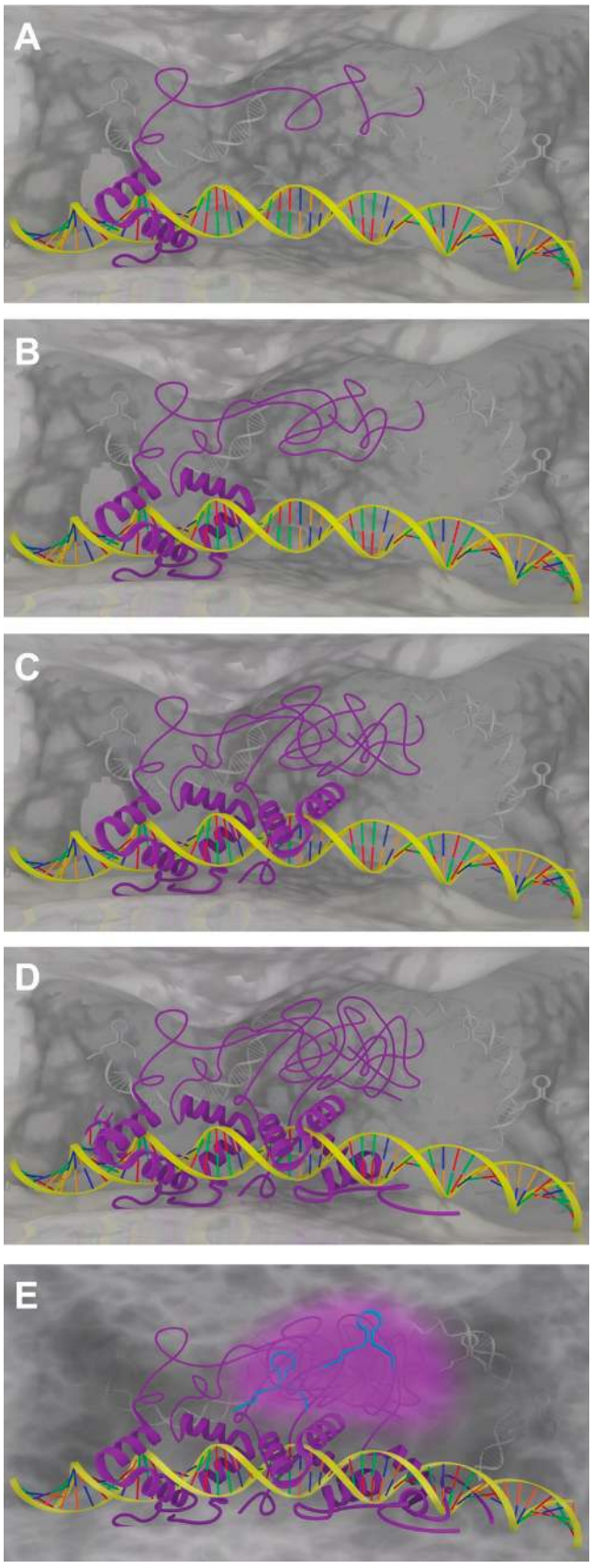

Figure 3. **DNA acts as a scaffold for EWS-FLI1 binding to GGAA repeats, leading to a putative phase transition based upon high concentration of EWS domains.** A series of panels (A–D) shows sequential binding of EWS-FLI1 (purple with helical region) to GGAA (red/green) repeats in the DNA. The high concentration of EWS domains that would occur as a result of multiple EWS-FLI1 proteins binding in a DNA microsatellite could lead to a phase transition based upon the intrinsically disordered low-complexity repeats. (E) The increased local concentration of these EWS domain subunits have emergent properties, at a critical concentration depicted here as five proteins, because of a phase transition leading to the sequestration of RNA (cyan). The assemblage and its interaction with RNA could be part of the transcriptional or posttranscriptional machinery. The capture of RNA could tether this dynamic phase separated assemblage to the nascent pre-mRNA or to the posttranscriptional splicing complex.

Malinovska et al., 2013). The carboxy terminal of EWS contains RNA-binding domains, homologous to those found in other RNA-binding proteins that form well-characterized assemblages (Han et al., 2012). The role of the intrinsically disordered low-complexity domains of EWS in the EWS-FLI1 fusion protein was discussed for many years (Üren et al., 2004; Ng et al., 2007). Recent evidence suggests that polymerization of the low-complexity domains forms an assemblage that can aberrantly recruit other cellular proteins (Kwon et al., 2013). This assemblage is postulated on the basis of the GGAA microsatellite repeats that would drive a high local concentration of EWS-FLI1 through interactions with the DNA-binding domain of FLI1 (Fig. 3). Both the EWS domain and the carboxy-terminal domain of FLI1 have low-complexity amino acid sequences that have the potential to create an assemblage at sites of transcription (Dunker and Uversky, 2010). Together with examples drawn from developmental biology, such as P granules (Feric and Brangwynne, 2013), and cancer biology such as TET proteins (Kwon et al., 2013), pathological assemblages may be dissolved (or allosterically inhibited) by peptides or small molecules that mimic or block the interactions that drive phase transitions.

Small molecules as probes of phase separation function

The discovery of the small molecule b-isox was serendipitous, but led to the identification of low-complexity intrinsically disordered motifs that mediate protein–protein interactions based upon phase transitions (Kato et al., 2012). In addition, IDRs are implicated in many diseases, from neuropathy to cancer. Therefore, there has been an effort to identify small molecules that could potentially disrupt assemblages and develop them as probes, with the goal of creating novel therapeutics (Metallo, 2010; Cuchillo and Michel, 2012). Direct screening of IDPs has been performed with either protein or a small molecule immobilized on a surface (Kemp et al., 2012; Hong et al., 2014a). Examples of the small molecule targets that involve intrinsic protein disorder include p53, c-MYC, EWS-FLI1, and CBFβ (Yin et al., 2003; Vassilev et al., 2004; Gorczynski et al., 2007; Follis et al., 2008; Erkizan et al., 2009; Mustata et al., 2009). These proteins all participate in canonical protein–protein complexes, and some appear to have emerging roles in assemblages. Thus, the pathological fibril formation that results from high local concentrations of these proteins and the dissolution potential of these fibrillar complexes in cells is a future challenge.

Many potential therapeutic targets involve proteins that are derived from chromosomal translocations (Mitelman et al., 2007). Chromosomal translocation fusion proteins, including EWS-FLI1 from ES, have been described as "undruggable" based upon their lack of unique structured domains (Üren and Toretsky, 2005). However, the first small molecule, YK-4-279, to directly target EWS-FLI1 with enantiospecificity inhibits EWS-FLI1 in both cell lines (Barber-Rotenberg et al., 2012) and xenograft models (Hong et al., 2014b). Future investigations will determine the extent to which YK-4-279 or other probes disrupt assemblages, and the degree of specificity with which this disruption occurs.

Future prospects

One of the significant challenges to understanding the structure and dynamics of assembly and disassembly of granules and other non-membrane-bound organelles is the difficulty of studying these assemblages in vivo. The ability to track multiple proteins in living cells, under various conditions that perturb their environment, is critical to study cellular phase separations. Spectacular advances have been made in methods for imaging individual proteins in cells (Sigal et al., 2007; Frenkel-Morgenstern et al., 2010; Farkash-Amar et al., 2012; Gebhardt et al., 2013). However, further developments will be required, including improved fluorescent probes and detection techniques (Coelho et al., 2013), to allow more precise localization of proteins within the cell and to obtain quantitative measures of their local concentrations if phase separations are to be studied in vivo. In addition, small molecule probes that disrupt protein interactions at the edges of a network, rather than by elimination of a network node, as occurs with RNAi, could provide critical tools for investigating assemblages in cells (for greater detail regarding network construction and utilization, see Zhong et al., 2009; Sahni et al., 2013). Investigation of assemblages using small molecules or peptides to disrupt specific protein interactions is likely to be highly informative and promises to provide insights into pathophysiology that are amenable to focused therapeutic targeting.

This review has described phase separation as an explanation for the coalescence of protein into assemblages. Moreover, we showed how low-complexity regions of IDPs are critically involved in the creation of many assemblages with diverse biological functions. Bioinformatic analysis showed that cells maintain aggregation-prone low-complexity proteins at levels below those required for aggregation through fine control of transcription, translation, and degradation (Gsponer and Babu, 2012). The use of biophysics to advance our understanding of assemblage is in its infancy. Technologies that can evaluate entire assemblages are emerging. Direct imaging of proteins based upon the limited resolution of light microscopy is improving, but probe and protein labeling remain a limitation (Huang et al., 2010). Reflected light sheet superresolution microscopy can visualize single proteins bound to DNA (Gebhardt et al., 2013; Zhao et al., 2014). To determine whether phase separation is occurring, intracellular rheology is able to measure phase changes in some cellular models (Deek et al., 2013; Stirbat et al., 2013). The combination of improvements to small molecule probes, imaging technology, and intracellular biophysical measurements will advance biology through a greater understanding of assemblages and their role in protein interactions. This enhanced ability to study and manipulate assemblages will lead to both deeper mechanistic understanding of biological processes and the ability to extend this knowledge in order to impact human health.

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The United States Patent and Trademark Office awarded a patent for YK-4-279 to Georgetown University; inventors include J.A. Toretsky. A license agreement has been executed between Georgetown University and Tokalas, Inc., for these patents, in which J.A. Toretsky is a founding shareholder and a consultant to Tokalas, Inc.

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