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Multivalent IDP Assemblies: Unique properties of LC8-associated, IDP duplex scaffolds

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Abstract

A wide variety of subcellular complexes are composed of one or more intrinsically disordered proteins (IDPs) that are multivalent, flexible, and characterized by dynamic binding of diverse partner proteins. These multivalent IDP assemblies, of broad functional diversity, are classified here into five categories distinguished by the number of IDP chains and the arrangement of partner proteins in the functional complex. Examples of each category are summarized in the context of the exceptional molecular and biological properties of IDPs. One type – IDP duplex scaffolds – is considered in detail. Its unique features include parallel alignment of two IDP chains, formation of new self-associated domains, enhanced affinity for additional bivalent ligands, and ubiquitous binding of the hub protein LC8. For two IDP duplex scaffolds, dynein intermediate chain IC and nucleoporin Nup159, these duplex features, together with the inherent flexibility of IDPs, are central to their assembly and function. A new type of IDP-LC8 interaction, distributed binding of LC8 among multiple IDP recognition sites, is described for Nup159 assembly.

Keywords

Multivalent intrinsically disordered proteins; protein scaffolds; multiple recognition motifs; self-association; dynamic complexes

Introduction

‘Intrinsic disorder’ is a collective term that embodies the ensemble nature and inherent flexibility of the structure of intrinsically disordered proteins (IDPs). The remarkable pleiotropy of IDP function arises from an essential feature of ensemble structure, namely, that each IDP chain samples numerous interconverting conformations among which the specific local structure, and the degree to which it is favored, is coded in the amino acid sequence [1]. The simplest conceptualization of an IDP must include a dynamic equilibrium

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among a multiplicity of conformations with varying degrees of order, and is irreducible to an 'average conformation that lacks order' [2–4]. Widely recognized characteristics of ensemble structure that are crucial to IDP function are: flexibility that confers versatility [5, 6], reversibility that confers ready responsiveness to local cellular changes [7, 8], and assortment of ligand binding sites along the IDP sequence that optimizes concerted pathways and cascades [9–11]. The very broad range of essential biological functions to which IDP ensemble attributes are adapted [3, 12–18] are illustrated in numerous regulatory pathways in which an IDP complex is a critical constituent, e.g., the Wnt signaling pathway [19], mitochondrial initiated cell death [20], regulation of eukaryotic cell division [21], and a DNA damage repair pathway [5].

Multivalent IDPs simultaneously bind multiple partner proteins and are well suited to large macromolecular assemblies [14, 22, 23]. Multivalent IDP assemblies encompass supramolecular complexes of one or more multivalent IDP chains with one or more partner ligands that tend to be folded protein domains. The partners may be different proteins and/or multiple copies of the same protein. A growing recognition of the ubiquity and functional significance of multivalent IDP assemblies is reflected in their burgeoning interest among researchers in protein-related fields. Functional adaptations of the physical attributes of IDPs, in general, are eloquently described and reviewed [13–15, 22, 24, 25]. LC8/IDP duplex scaffolds constitute one category of multivalent IDP assemblies. To place IDP duplexes in context within the larger field, we developed a general classification scheme for multivalent IDP assemblies based on a few simple structural criteria, i.e., the number of IDP chains and the arrangement of partner proteins involved in forming a functional complex (Fig. 1). In this review we discuss the unique structure/function properties of IDP duplex scaffolds, that is, features duplexes possess in addition to the many other remarkable features they have in common with single chain scaffolds. LC8/IDP duplex scaffolds function as core components in a broad array of cellular assemblies, each employing a different IDP and coordinating the collective activity of bound IDP partner proteins. Example IDPs in duplex scaffolds include IC in the dynein cargo domain [26, 27], Nup159 in the nuclear pore [28], zinc finger protein ASCIZ [29], signal transduction protein Kibra [30], RNA-binding protein Swallow [31], mitotic protein Chica [32, 33], and rabies virus phosphoprotein.

The effort in our lab to characterize one category of multivalent IDP assemblies – polybivalent IDP duplex scaffolds – has led us to seek a general classification scheme based on a few simple criteria, i.e., the number of IDP chains and the arrangement of partner proteins involved in forming a functional multivalent IDP assembly, and to explicate IDP duplex scaffolds vis-à-vis other categories of multivalent IDP assemblies.

In Fig. 1 we offer such a scheme. The discussion includes a brief summary of representative examples of each category along with a more extended analysis of features unique to polybivalent IDP duplex scaffolds, the category in which a duplex –formed by association of two IDP chains with the protein LC8– presents multiple sites for binding bivalent partners.

Categories of multivalent IDP assemblies

We group multivalent IDP assemblies into A) Binary complexes, B) IDP single chain scaffolds, C) IDP duplex scaffolds, D) Higher order IDP associations, and E) IDP multi-site collective binding ligands. These five types are illustrated in Fig 1, and discussed in order below.

Binary Complexes

Binary complex assemblies (Fig. 1A) consist of an IDP chain with several recognition motifs of variable length along its sequence that specifically interact with different sites on the surface of one folded partner. This group represents coupled binding and folding, a well described process in which an IDP binds several sites on a folded domain and in the process acquires a three-dimensional structure [34–38]. In folding-upon-binding, multiple IDP binding sequences interact specifically with as many folded domain sites, often distributed distally on the folded surface. This interaction mode is highly specific, as well as easily reversible in response to changes in cellular requirements. Two well known examples are illustrated in Fig. 1A (upper frame), which depicts the interactions between protein phosphatase 1 (PP1) and intrinsically disordered regions (IDRs) of spinophilin (top) and protein inhibitor-2 (bottom). Each directs substrate specificity by blocking one of the three PP1 substrate binding sites. The intrinsically disordered nature of these two proteins allows them to “wrap around” PP1 and binds sites on different faces of the protein and cover one or more substrate binding sites [39, 40].

Another example in this category (Fig. 1A (2)) is Keap1, a hub protein that modifies its intrinsically disordered binding partner Nrf2 through a proposed “hinge and latch” process [41, 42], leading to its polyubiquitination and subsequent degradation. The high-affinity site (blue) retains the ligand and acts as a hinge, while the weak-binding site (yellow) serves as a latch that operates only when Keap1 is in a specific conformation that is dependent on the cellular redox environment [42, 43]. Disorder and flexibility allow the weak-binding site frequent contact with the second, unoccupied binding site on Keap1 (Fig. 1A(2), grey arrow).

IDP Scaffolds

IDP scaffolds, (Figs. 1B and 1C), present a series of recognition motifs distributed along their sequence to a suite of partner proteins. Assortment of bound partners along the IDP facilitates the concerted operation of multiple components in a common function, along with efficient integration of regulatory proteins. Each motif is short, about 8–10 residues, and when bound, is incorporated into the existing fold of the partner as an element of secondary structure flanked by disordered linkers.

IDP scaffolds attached at intervals to partner proteins are dynamic and flexible assemblies, in flux with numerous cell components in a tightly packed cellular environment, poised for specific and reversible interactions with an ever-changing pool of reactive partners and their regulators. Pleiotropic function, ubiquitous distribution in intracellular systems, concerted action, fast response to changes in cellular needs, modulation by reversible binding of

regulators, and participation in alternative pathways to the same end, are all recognized aspects of IDP scaffold structure/function relationships [15]. As these remarkable properties are shared by both single chain and duplex IDP scaffolds, we ask what *additional* features are offered or displayed only by duplex scaffolds. We answer this question in the context of a general overview of IDP duplex scaffolds with specific examples from our lab, illustrated in Fig. 1C.

IDP Single Chain Scaffolds

The most numerous and well described group of IDP scaffolds contain one IDP chain bound to several or many partner proteins, Fig. 1B. The intrinsic disorder of the IDP assists catalytic or signaling pathways by spatially and temporally integrating essential functional components. The limited space allotted here to single chain IDP scaffolds does not reflect their relative importance, but rather that excellent reviews of this broadly distributed and functionally essential category are readily available in the literature [15, 17, 44, 45].

A representative example of a disordered scaffold punctuated with short linear motifs is RNase E, an essential endoribonuclease conserved across many bacterial phyla [46]. Each RNase E chain binds three partner proteins whose collective action is important in RNA degradation and turnover and in RNA precursor processing [47]. The endoribonuclease activity of RNase E resides in the structured N-terminal half of the protein, while the intrinsically disordered C-terminal region serves as a scaffold for the prokaryotic RNA degradosome consisting of three proteins: the DEAD-box RNA helicase, the glycolytic enzyme enolase, and the exoribonuclease (different grey shapes) [48–50]. RNase E is an unusual scaffold in that its binding partners do not directly interact with each other [48], but rather, they function in sequence on a flexible scaffold that brings the three proteins into close proximity.

IDP Duplex Scaffolds

IDP duplex scaffolds, Fig. 1C, are composed of two IDP chains in parallel alignment, connected by one or many bivalent partner proteins and/or by inter-chain interactions of identical sequence segments. Specific cross-linking sites are distributed along each IDP chain, and are either motif sequences or self-association sequences. Each motif (yellow sphere) recognizes a bivalent ligand (bi-lobed shape). Self-association sequences (pink) interact in the duplex to form inter-chain, dimeric domains.

While IDP duplex scaffolds share many extraordinary properties with single chain scaffolds, as outlined above, they also display unique features, namely: 1) parallel alignment of two IDP chains cross-linked by reversible, non-covalent protein-protein interactions; 2) formation of new ordered, self-associated domains resulting from interactions of identical sequences within each chain; 3) enhancement, relative to the same IDP in monomeric form, of binding affinity for additional bivalent ligands and of self-association tendency, and, 4) ubiquitous binding of LC8, a folded, bivalent protein first described in dynein. All four features are observed in all currently reported IDP duplexes, and new examples in the literature are steadily accruing. The growing range of biological functions served by IDP duplex scaffolds is impressive, from dynein assembly and regulation [26, 51–53], to nuclear

pores [28, 54], to virus maturation [55, 56]. Additional examples are associated with mRNA localization and lung development [31, 57, 58]. While the full functional implications of IDP duplexes are still under examination in our lab and others, these four intriguing and unique characteristics of IDP duplex structure are well documented.

- 1) Parallel alignment – in-register cross-linking – is the combined result of symmetrical binding of a bivalent ligand(s) and of dimerization of self-association sequences. Since there are typically multiple binding sites for binary partners along the IDP sequence [26, 28, 59], it is conceivable that cross-linked isoforms could occur if, for example, a partner binds one chain at motif number 1 and the second chain at motif number 2; but such out-of-register species have not been observed.
- 2) Self-association domains of IDP duplexes cross-link the duplex along with binary partner proteins, and importantly, they *mediate the specificity of an IDP sequence for duplex formation* [52]. Once the duplex is formed, self-association domains also *extend* the specificity of an IDP sequence for regulatory and/or accessory ligands to other ligand types. This is accomplished by formation of new ordered surfaces that may bind additional ligands, including those that are monovalent.

In the absence of partner proteins, apo ensembles of IDPs involved in duplex scaffolds are equilibrating populations of monomers and dimers. For some, the equilibrium favors monomers while for others the ensemble favors dimers; the relative monomer/dimer population of self-association domains presumably reflects the tendency of a given self-association sequence to engage inter-chain IDP interactions. In one well-characterized example, the dynein intermediate chain (IC), the apo ensemble favors monomers and also displays nascent order within the self-association sequences [26, 51, 52, 60]. In another case where the apo ensemble favors dimers, such as in Swallow and Myosin V [31, 61, 62], the self-association domains interact but retain conformational heterogeneity and flexibility. In a third example such as rabies phosphoprotein P [63], the self association sequences interact strongly and fold into highly ordered domains amenable to X-ray crystallography.

The ensemble nature of IDP structure means that tight binding of a new ligand to the self-association domain does not require that the major conformational population have high affinity self-association sequences. An ensemble that weakly favors self-dimerization can still bind specific dimer-targeted ligands by conformational selection. A notable consequence is that self-associating domains may constitute new targets for drugs directed against pathological IDP duplexes (e.g. RNA viruses [55, 56]) whether or not the self-association domain favors stable, folded dimers – any dimeric conformation in the ensemble could be ‘selected’ by the drug, and so shift the equilibrium to that conformation.

- 3) Bivalency refers to the entropic enhancement of an additional binding event; it may be conceptualized as the increase in local effective concentration of motifs or self-association sequences or as an entropic ‘pre-payment’ of numerous degrees of freedom lost when two chains are constrained in a cross-linked duplex [26, 52, 53, 64]. Bivalency in the dynein IC duplex is discussed below.

- 4) Cross-linking by at least one LC8 is *always* observed in the known IDP duplex scaffolds. Two IDP chains bind LC8, one in each of two grooves near the LC8 dimer interface. The crystal structure of the complex of LC8 and two copies of a peptide corresponding to the LC8 recognition motif in the IDP sequence is shown in. The motif peptides are aligned in parallel, and each is incorporated as a new strand in the existing β -sheet of LC8. In one IDP duplex, dynein intermediate chain discussed below, additional cross-linking proteins are observed [45] and in other duplexes, multiple LC8s bind along the IDP chain [28, 59, 65, 66]. While an IDP duplex scaffold may bind other bivalent partners, e.g., Tctex1 and LC7 with dynein IC [26, 53], only LC8 occurs in every IDP duplex reported thus far.

In summary, the unique characteristics of IDP duplex scaffold structure – parallel alignment, self-association domains, bivalency, universal cross-linking by at least one LC8 – are mutually reinforcing and interdependent. For example, cross-linking at two segments (self-association domain *and* bivalent partner motif) promotes in-register alignment, parallel alignment promotes bivalency, and bivalency promotes binding of additional bivalent ligands, and so on. The unique characteristics of IDP duplex scaffolds, while ultimately a manifestation of the inherent properties of IDPs, originate in their unique duplex organization.

Higher Order IDP Associations

IDP assemblies composed of higher order self-associated complexes, Fig. 1D, can reversibly incorporate and/or release other functional proteins. Often the assemblies undergo a phase transition and form a hydrogel, and act as a subcellular organelle unbounded by a membrane [67, 68]. A characteristic feature of these IDPs is low complexity sequences of phenylalanine-glycine, polyglutamine, or [G/S]Y[G/S] repeats [69–72], that often interact with homotypic and heterotypic proteins containing the same repeat [73, 74]. Intermolecular affinities result in networks of multivalent interactions forming an aggregate that is phase-separated from the surrounding solution (e.g. liquid droplets or hydrogels). The yeast germ cell RNP granules are one such example where these membrane-less organelles provide a means for cellular polarization of RNA during cell division [75].

Multi-site Collective Binding

Fig. 1E illustrates an IDP in which multiple sites of a polyvalent ligand collectively engage a single site of a partner protein. This unusual binding mode involves interactions of the folded partner with various binding sites along the IDP chain. This category is represented at present by a polyphosphorylated IDP, in which several phosphate sites interact collectively with one site on a receptor [76].

LC8 Cross-linking of IDP Duplex Scaffolds

In duplex scaffolds, the IDP is specific to the system, but in all known examples, LC8 is a cross-linking partner protein. As such, LC8 has been termed an IDP dimerization ‘hub’ protein [77], and its broad occurrence indicates its central cellular functions. The name LC8 was coined in dynein literature to abbreviate ‘light chain 8’ of the dynein cargo attachment

domain. Before the LC8 hub hypothesis it was common to assume that LC8 serves primarily as an adaptor molecule connecting dynein to dynein cargo. Thus, proteins that are LC8-associated were commonly assumed to function in conjunction with dynein transport of cargo. Since then, the hub idea has had numerous corroborating examples. In all LC8-IDP complexes for which structural information is available, around a dozen thus far, LC8 cross-links an IDP duplex scaffold in diverse functional systems with no proven dynein association.

The number of LC8 dimers in an IDP duplex spans the gamut, varying from one in Swallow and rabies phosphoprotein [56, 62], to several in Chica and nucleoporin Nup159 [28, 32], to many in ASCIZ (ATM-Substrate Chk Interacting Zn²⁺ Finger) [59]. Interestingly, ASCIZ functions as an LC8 transcription factor, and it has been proposed that LC8 binding to ASCIZ mediates the level of LC8 expression in the cell [29].

In principle, IDP duplexes cross-linked by bivalent proteins other than LC8 are possible but not yet identified, since many new IDP duplexes are initially recognized as LC8-binding proteins. However, the universality of LC8 as the IDP duplex crosslink is consistent with the example of rabies phosphoprotein, an IDP and one of only five proteins coded by the virus genome, which recruits host LC8 rather than any other cross-linking protein [56], and with the proposed LC8 self-regulation of its own expression [76].

In crystal structures of LC8 bound to short linear motifs from several different IDPs [78–80], e.g. Fig. 3, all motifs interact with residues in identical symmetric grooves of the LC8 dimer. This binding versatility is ascribed to the flexibility of LC8 in that region [79, 81, 82]. The core of the LC8 homodimer is composed of a 12-stranded β -sandwich where each monomer contributes 5 β -strands and the sixth β -strand is formed by the bound peptide. The antiparallel β -sheet forces antiparallel binding of the IDP partner and therefore parallel orientation of the two IDP chains [78, 79]. While the LC8 consensus motif is identified [83] as an 8- to 10-residue sequence called a TQT, SQT or QT motif, not all such motifs bind LC8 [28, 54], and some motifs known to bind LC8 do not contain glutamine or threonine [84]. The variation of LC8 motif sequences suggests that involvement of LC8 in IDP duplexes has a long evolutionary history. If so, LC8 itself is expected to show some measure of evolutionary sequence divergence, and this is observed to a modest extent in LC8 sequences from yeast and higher organisms, and in the structural relatedness of LC8 to another dynein subunit, Tctex1 [26], a structural homolog of LC8 that binds a second near-by motif on dynein intermediate chain IC. In vertebrate IC, the TQ motif is present in both Tctex1 and LC8 recognition motifs (SKVTQV and SKETQT in *Danio rerio*) suggesting that the ancestral IC was bound to two LC8 dimers. Indeed in yeast, two LC8 dimers (Dyn2 in yeast) bind near-by motifs on IC [85].

Given the flexibility and disorder of IDPs, study of the molecular bases of duplex function requires a multidisciplinary approach. For this, we integrate NMR spectroscopy for study of residue level interactions of flexible domains, X-ray crystallography for atomic level structure of stable complexes, and isothermal titration calorimetry for determination of binding energetics. 13 crystal structures of LC8 bound to peptides have been solved [78–80, 83, 84, 86–90], revealing features of the LC8 recognition motif that confer affinity and

specificity. NMR studies of the full-length LC8 binding domain have been performed for multiple binding partners [9, 28], shedding light on the transient structure and dynamics of these regions. A cryo-EM image of Nup159, showing 5 LC8 homodimers bound to two Nup159 chains in parallel has also been published and, remarkably, the rigid scaffold is so stable that it has been used as a marker in cryo-EM experiments [91]. Additionally, *in vivo* studies of LC8-IDP duplexes are complicated by the ubiquitous nature of LC8 and the presence of multiple LC8 binding sites. Mice deficient in LC8 exhibit developmental defects [58, 92] and *Drosophila* with reduced LC8 levels die in early embryonic stages of development [93, 94]. Many LC8 binding partners contain multiple binding sites that must be fully removed in order to study the functional impact of LC8 binding *in vivo*, which becomes challenging when there is ambiguity in identifying all binding motifs. Below, we summarize the results of our structure/function studies of two LC8-IDP duplexes that integrate many of these approaches: dynein intermediate chain IC and nucleoporin Nup159.

Dynein IC Duplex Scaffold

Dynein intermediate chain IC is a core component of the cytoplasmic dynein cargo attachment domain. LC8 binding to apo IC dimerizes IC to form an IDP duplex scaffold (illustrated in Fig. 1C, frame (1), and Fig. 2) [26]. IC contains adjacent recognition motifs for LC8 and for Tctex1. The ternary complex is a stable polybivalent scaffold in which an LC8 dimer and a Tctex1 dimer each bind the same two IC chains. In the absence of binding partners, apo IC constructs are predominantly monomeric, with nascent order in the self-association domain [60, 95, 96]. When either LC8 or Tctex1 is bound to apo IC, affinity for the other is enhanced 50-fold relative to apo IC, due to a bivalency effect, namely, a reduced entropic penalty for the second binding event [26]. Similarly, binding of LC8 to apo IC enhances self-association interactions in a region of IC that is 84 residues away from the LC8 recognition motif, in the C-terminal helix self-association domain (pink) on the rightmost duplex of Fig. 1C, frame (1). A bivalency effect is also observed when an artificial cross-link (inserted disulfide) is engineered at this site; in disulfide cross-linked IC, LC8 binding is enhanced 6-fold [52]. Not every duplex cross-link results in binding enhancement of other ligands. Dimeric light chain 7 (LC7) does not enhance overall LC8 binding affinity relative to monovalent IC, and *vice versa*, [52], possibly because LC7 binds to residues in the self-association domain and so disrupts self-association interactions.

The coupling of LC8 binding and IC self-association suggests that in the apo IC ensemble a small population of self-associated dimer has bivalency-enhanced LC8 affinity and so LC8 selectively binds this conformation, and initiates the assembly process. Then Tctex1 binding further stabilizes the duplex, which subsequently binds LC7. Later binding of LC7 to an already stabilized duplex is expected because LC7 disrupts the self-association domain and folds those residues into an LC7-bound helix, as shown by a crystal structure [52]. In the LC7-bound duplex, disruption of IC self-association interactions are apparently compensated by the combined effects of bivalency and of formation of new transient IC-IC contacts detected by fluorescence energy transfer when both LC8 and LC7 are bound but not when only one is bound [52]. With LC8 bound, IC-IC self-association involves one helix from each chain, presumably packed against the other as in Fig. 1C. With LC7 bound, these residues are folded into the LC7 structure, as described above. With both LC8 and LC7

bound, transient IC-IC interactions stabilize the linker between LC8 and LC7 (Fig. 2, and legend). This conformational versatility of IC segments resides in the inherent flexibility and adaptability of IDP complex ensembles.

Thus, the assembly of two IC chains into a flexible polybivalent scaffold is modulated by long range coupling between IC self-association and LC8 binding and by subsequent binding of multiple additional bivalent partners. In its fully bound state, this polybivalent scaffold remains partially disordered and therefore structurally pliable [9, 52]. In addition to providing a duplex scaffold for the three dimeric dynein light chains, IC also interacts directly with components of several protein complexes (Dynactin, NudE, and RZZ) that regulate the function and activity of the dynein motor complex in the cell [97], and with the protein Huntingtin, implicated in Huntington's Disease [98].

Among the regulatory proteins that interact with IC, we are particularly interested in dynactin, which is critical in normal cellular functions [99], and in NudE, a 'nuclear distribution' protein that is essential in diverse processes including kinetochore and centrosome migration [100, 101]. Dynactin and NudE are localized with dynein in cellular compartments and they both bind to proximate regions on IC and with similar *in vitro* affinity, with NudE binding to region 1 but dynactin binding both regions 1 and 2 (Fig. 2) [9, 51]. The coordination of their binding to IC is therefore a pertinent question. We have proposed that events that modify region 2 (brown), but do not significantly affect region 1 (orange), could regulate dynactin binding, but have limited effect on NudE binding [51, 102]. Region 2 is close to a disordered linker that has phosphorylation sites, suggesting that phosphorylation events may modulate binding of NudE *versus* dynactin (Fig. 2). In summary, disorder in apo IC is an integral part of its assembly, and disorder retained in the IDP duplex is an integral part of its function and regulation of cargo binding and transport.

Considering its many interactions with light chains, regulatory molecules, and putative dynein cargo, IC appears to be a key modulator of dynein assembly and attachment to cargoes. Since the majority of these interactions are localized to the N-terminal 300 amino acid segment which is largely disordered [96] and is rich in phosphorylation and alternative splicing sites [103, 104], the disordered regions of IC therefore figure importantly in the functional versatility and binding-partner diversity of the entire complex.

Nup159 Duplex Scaffold

The LC8-Nup159 duplex scaffold illustrates IDP duplexes that are incorporated into multicomponent subcellular structures, in this case the yeast nuclear pore complex (NPC), a 60 MDa complex that directs nucleocytoplasmic transport. One essential module of the NPC is the Nup82 subcomplex, which is located on the cytoplasmic side of the NPC and is involved in nuclear mRNA export. The Nup82 module is comprised of Dyn2 (the yeast LC8 ortholog), Nup159, Nup82 and NSP proteins [105]. Nup159 is a 159 kDa protein whose sequence is predicted to be a β -propeller at the N-terminal end, followed by a long segment of FG repeats lacking secondary structure, then by the Dyn2 interacting domain (DID) sequence bearing multiple recognition motifs for Dyn2, and at the C-terminus a predicted helical segment that includes coiled-coil motifs. The DID between the FG repeats and the

predicted coiled-coil forms a duplex of 2 Nup chains cross-linked by 5 Dyn2 dimers, as diagrammed in Fig. 1C, frame (2).

In a series of constructs of the DID containing increasing numbers of Dyn2 recognition motifs, isothermal titration calorimetry (ITC) shows that when one motif is present, Dyn2 binding is very weak, but when two motifs are present, the average affinity is significantly increased, indicating cooperative binding due to bivalency [28]. Similarly, when three recognition motifs are present, fits to the single ITC binding curve give an average Dyn2 affinity that is significantly higher than when only two motifs are present. When the fourth and fifth recognition motifs are included in the constructs, however, the trend is ended, and the average affinity is slightly decreased.

In full length DID, with all motifs present, ITC data show a stoichiometry of 5 Dyn2 per duplex in the presence of excess Dyn2. But at substoichiometric ratios of Dyn2:Nup159, there is no Dyn2 binding preference for one motif *versus* another, resulting in a mixture of partially bound duplexes [28]. This is inferred from NMR titration methods that monitor involvement of specific residues in Nup159-Dyn2 binding interactions; in the titration experiments, loss of intensity is measured for samples with increasing Dyn2:Nup159 ratios. Diminished intensity of backbone NH peaks indicates direct involvement of that residue in Dyn2 binding and/or restricted local motion due to proximate binding. We observe lower peak intensity along the entire DID sequence even at Dyn2 concentrations equivalent to one Dyn2 dimer per DID duplex [47, Clark & Barbar, unpublished data], meaning that binding of the first Dyn2 molecules occurs at multiple sites.

This distributed binding of Dyn2 among the five Nup159 motifs is reminiscent of the multisite collective binding of Fig. 1E, where multiple phosphorylation sites on an IDP apparently interact transiently with the binding pocket of the partner protein, resulting in a dynamic ensemble of IDP/ligand complexes. While this system has some similarity to the Nup-Dyn2 complexes at substoichiometric ratios of Dyn2, there is a significant difference: in functional Dyn2-Nup159 duplexes, all IDP sites are *fully* bound to ligand. Thus, the multi-site binding of Dyn2 is more a distributed binding process, rather than a collective binding mode. As more Dyn2 dimers are added, their binding is distributed all along the duplex until, ultimately, the assembly attains the final ratio of 5 Dyn2 per duplex.

Distributed binding of Dyn2 retains Nup159 flexibility in ensembles of partially cross-linked duplexes, and we expect that this is essential to the biological activity of Nup159. A possible function of distributed Dyn2 binding is to maintain flexibility at lower Dyn2 concentrations before the final assembly of five Dyn2 in the Nup159 duplex that is apparently quite rigid and thus unfavorable energetically, i.e. of very low entropy. Distributed binding could optimize the system-wide energetics as increasing numbers of Dyn2 are added by averaging the entropic penalty over numerous binding reactions, and maximizing the bivalent affinity enhancement of various Nup159 motifs for Dyn2.

The expectation that the fully bound Dyn2-DID complex is rigid comes from electron microscopic studies of nuclear pore formation [50]. In the Dyn2-DID complex, a ~20 nm long rod-like structure is observed in electron micrographs of the DID domain [54]. In these

structures five Dyn2 dimers are stacked like beads on two Nup159 strands and the rigidity of this parallel arrangement is suggested to help protrude Nup159 FG repeats and the N-terminal β -propeller domain toward the central transport channel [28, 54]. Thus it appears that the Dyn2-Nup159 duplex is a key organizing component of the NPC complex, directing FG repeats towards the central transport channel to form a cargo-accessible domain. Consistent with the proposal that the functional adaptation of multiple LC8 binding sites is distributed binding, our ITC data show that DID constructs having either the last two motifs or the first three motifs form a maximally stable complex [28], and therefore the evolutionary selection of five Dyn2 cross-links is apparently not for overall stability.

In addition to distributed binding, Nup159 has a sequence near the DID that is crucially involved in pore formation, and that appears to be a self-association domain; this is significant because self-association, alignment and duplex stability are mutually reinforcing. Recent structural data for a reconstituted Nup82 module suggest that the C-terminal portion of Nup159, which includes the DID and the C-terminal helices, forms the structural backbone of the complex, along which the other subunits – Nup82, Nsp1, and Dyn2 – become organized, and that this segment of Nup159 is required for organization of the whole complex [105]. Critical to this process is a short, weakly predicted coiled-coil sequence separated from the DID domain by a flexible linker, and shown *in vivo* to be essential for NPC assembly [98]. Since IDP self-association and LC8 binding are mutually reinforcing, the weakly predicted coiled-coil sequence in Nup159 likely forms a self-association domain in the duplex. The *in vivo* demonstration that the weakly predicted coiled-coil sequence adjacent to the DID is essential for NPC assembly suggests that in the duplex these residues form a self-association domain which plays a critical role in nuclear pore assembly.

In summary, the thermodynamics of Dyn2 binding to Nup159, taken together with NMR titration experiments of Nup159 with Dyn2, implies that the essential involvement of the Nup159-Dyn2 complex in nuclear pore formation arises from unique properties of the IDP duplex scaffold. These include self-association and distributed binding, the latter a new process in multivalent IDP assemblies. We expect that, in general, for IDP duplexes having multiple LC8 motifs, distributed binding underlies the function of multiple LC8 cross-links. In the Nup159-Dyn2 duplex, distributed binding is apparently a novel adaptation of IDP flexibility to produce duplex rigidity.

Future Directions for IDP duplex scaffolds

Current studies of multivalent IDP duplex assemblies tend to focus on IDP-partner protein interactions. It is clear that duplex scaffolds align in parallel, form self-associated domains, display bivalent enhancement of affinity for additional ligands, and are always cross-linked by LC8. Other aspects of growing interest in IDP duplexes are the processes associated with flexible sequence segments, or linkers, between IDP binding sites for partner proteins. Flexible linkers often connect essential functional domains, and as such are well placed to host novel regulatory processes such as competition of regulatory molecules for overlapping binding sites [9, 44, 92], alternative splicing, and posttranslational modification. An example of a regulatory effect from posttranslational modification, is in the dynein IC duplex where

phosphorylation in a disordered linker abolishes IC binding to the regulator dynactin [102]. An example in the Nup159 duplex is the linker between the DID and a self-association domain, which when deleted, abolishes binding of Dyn2 to DID *in vivo* even though the DID domain is left intact [105]. Flexible linkers also contain dynamic self-association domains that acquire alternative ordered conformations, as in IC segments that make up the binding site of LC7 [44]. Thus, although only a handful of IDP duplex scaffolds are well characterized thus far, upcoming molecular-level studies promise new structure/function advances since, remarkably, IDP duplexes participate in essential cellular functions in over 100 diverse systems [77, 83, 106].

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Abbreviations

IC	dynein intermediate chain
LC8	dynein light chain 8
LC7	dynein light chain 7
Tctex1	An LC8-like dynein light chain
Dyn2	LC8 in yeast
DID	Dyn2 interaction domain
PP1	protein phosphatase 1
I-2	inhibitor 2
Keap1	Kelch ECH associating protein 1
Nrf2	nuclear erythroid 2-related factor 2

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Figure 1. Multivalent IDP assemblies. Five categories are grouped by the number of IDP chains and arrangement of partner proteins

(A) Binary complexes composed of one multivalent IDP and one binding partner. (1) Folding-upon-binding. PP1 (grey shape, left) binds either of two IDPs, spinophilin (upper chain) or protein inhibitor-2, I-2, (lower chain). Upon binding to PP1, spinophilin undergoes a “disorder to order” transition and forms two β -strands (yellow) and an α -helix (pink) at three PP1 contact points. In the coupled folding and binding of I-2, the IDP wraps around PP1 to form an extended α -helix (pink), and incorporates a loop structure that brings exposed phosphorylation sites Ser86 and Thr72 (white spheres) into close proximity, and subsequently turns on PP1 phosphatase activity. (2) The disordered domain of Nrf2 (aqua chain) has two binding motifs (blue, pink) for Keap1 (grey). In a proposed “hinge and latch” process, Keap1 binding to the first Nrf2 site exposes six lysines in an α -helix (pink) located between the two motifs. Keap1 binding to both sites results in polyubiquitination (white

spheres) of the helix (pink), and proteolysis of Nrf2. **(B)** Single IDP chain scaffolds. In this illustration a generalized multivalent IDR (aqua) with three linear motifs (sphere, rod, triangle) binds three partner proteins (cylinder, bi-lobed shape, rectangle) and facilitates interactions between the proteins in a concerted series of reactions. The intrinsically disordered region is part of a larger protein that also has a folded domain (blue pear shape). **(C)** Duplex IDP scaffolds consist of two IDP chains having at least one LC8 cross-link, and sites for other bivalent ligands and/or self-association domains. (1) Dynein IC binds LC8 and Tctex1 to form a scaffold (rightmost structure) with an ordered self-association domain (pink) and additional bivalent binding sites (not shown). Binding of one dimer, LC8, to the intrinsically disordered intermediate chain enhances the binding of the second dimer, Tctex1, and vice versa. (2) Nup159 DID chains (blue, at left) have six apparent LC8 motifs (yellow spheres) but binds five copies of yeast LC8 (Dyn2) to form a rigid duplex (rightmost structure). At lower molar ratios of Dyn2:Nup159, there is distributed binding of LC8 among the motifs, as depicted in the center brackets by the three flexible duplex species. **(D)** Higher order association of polyvalent IDPs can induce a phase change to form a large network of chains bound together by multivalent interactions. Shown is the formation of a protein hydrogel, as in wheat germ cell RNP granules, when repeats in the C-terminal domain of the protein self-associate. **(E)** In collective binding, a multivalent IDP interacts with one binding partner in a dynamic process (depicted in brackets) in which multiple IDP sites interact transiently and repeatedly with a single site on the partner. In the example shown, a single site on the receptor Cdc4 (crescent shape) interacts with multiple phosphorylated sites on the disordered cyclin-dependent kinase inhibitor (aqua chain). References and further discussion of the five categories are given in the text.

Figure 2. Schematic representation of cytoplasmic dynein IC duplex and two regulatory proteins (*left*) The dynein complex of intermediate chain, IC (blue), and heavy chain (grey). The 300 amino acid N-terminal domain of IC (aqua chain) is intrinsically disordered and monomeric except for a short single α -helix (red). The C-terminal domain of IC (light blue shape), predicted to be ordered, binds the tail of the heavy chain (light grey). In the motor domain of the heavy chain, six spheres represent the sites for ATP hydrolysis. (*middle*) Three homodimeric light chains Tctex1 (green), LC8 (blue), and LC7 (purple) bind IC and form a duplex. We hypothesize that LC8 binding initiates the duplex formation. Transient IC-IC contacts, present when both LC8 and LC7 are bound, are indicated by short segments of close, parallel blue chains. The IC duplex is essential for formation of the dimeric motor domain that is necessary for dynein activity [88, 107]. Light intermediate chains (LIC) are dark grey. The N-terminal domain of IC also binds dynein regulator proteins NudE (light orange, bi-lobed) and dynactin (light yellow, bi-lobed); both are dimeric coiled-coils that bind to residues in the helical region (red). NudE binds only to Region 1 (orange bar), while dynactin binds to Regions 1 and 2 (brown bar), although the overall IC affinity is similar for both. Regions 1 and 2 are separated by a short flexible linker. The resulting IC duplex contains other disordered linkers, one separating Region 2 from the Tctex1 binding site, and another separating the LC8 binding from the LC7 site. (*right*) Phosphorylation of the disordered linker between Region 2 and the Tctex1 binding motif prevents dynactin binding [108].

Figure 3. Crystal structure of LC8 bound to two recognition motif peptides

Dimeric LC8 (light and dark blue monomers) forms a complex with two copies of a peptide (yellow) corresponding to an LC8 recognition sequence. The peptides are aligned in parallel, and each is incorporated as an additional β -strand in the β -sheet of an LC8 monomer subunit. In the LC8/IDP complex, one recognition motif on each IDP chain binds LC8, as in Figures 1C and 2.