REVIEW

Spatial and temporal organization of multi-protein assemblies: achieving sensitive control in information-rich cell-regulatory systems

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The regulation of cellular processes in living organisms requires signalling systems that have a high signal-to-noise ratio. This is usually achieved by transient, multi-protein complexes that assemble cooperatively. Even in the crowded environment of the cell, such assemblies are unlikely to form by chance, thereby providing a sensitive regulation of cellular processes. Furthermore, selectivity and sensitivity may be achieved by the requirement for concerted folding and binding of previously unfolded components. We illustrate these features by focusing on two essential signalling pathways of eukaryotic cells: first, the monitoring and repair of DNA damage by non-homologous end joining, and second, the mitotic spindle assembly checkpoint, which detects and corrects defective attachments of chromosomes to the kinetochore. We show that multi-protein assemblies moderate the full range of functional complexity and diversity in the two signalling systems. Deciphering the nature of the interactions is central to understanding the mechanisms that control the flow of information in cell signalling and regulation.

Keywords: structural biology; protein assemblies; cell signalling

1. Introduction

There is estimated to be one billion individual protein molecules present in mammalian cells at one time, of which up to 10 per cent are involved in cell signalling [1]. In this crowded environment, cell signalling and regulation have evolved to depend on multi-protein complexes that are specific and reversible in order to achieve high signal-to-noise. Structural biology is uniquely placed to provide an insight into how this is achieved. Here, we illustrate these themes by

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Dedicated to Alan L. Mackay, who has taught us so much about the assembly, organization and function of complex multi-component systems.

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focusing on two examples of such regulatory processes, DNA repair and mitotic checkpoint control, which comprise essential surveillance pathways that monitor and provide the time-for-error correction of key events of the cell cycle.

Non-homologous end joining (NHEJ), which is one of the two major pathways responsible for repairing double-strand breaks (DSBs) in DNA, features three main steps in vertebrates: synopsis; end processing; and end joining [2,3]. Synapsis brings the two DNA ends into close proximity and is carried out by the DNA-dependent protein kinase complex (DNA-PK), comprising the Ku70/80 heterodimer and the DNA-PK catalytic subunit (DNA-PKcs) [4,5]). End processing involves an array of nucleases to cut DNA overhangs and a polynucleotide kinase/phosphatase for preparing damaged DNA ends ready for ligation [6]. Finally, the two ends are joined by the ligase complex, including DNA ligase IV (LigIV), X-ray cross-complementation group 4 (XRCC4) and XRCC4-like factor/Cernunnos (XLF) [7,8].

The mitotic spindle assembly for the checkpoint (SAC) is the evolutionarily conserved regulatory mechanism that ensures the maintenance of genomic stability in higher organisms. The SAC controls the timely and accurate segregation of chromosomes by delaying the onset of anaphase until all chromosomes are properly bioriented and attached to the mitotic spindle. The serine/threonine kinase Bub1 is essential for the assembly of the functional inner centromere and mediates the recruitment of other checkpoint components in cells that have the checkpoint unsatisfied, while another serine/threonine kinase, BubR1, associates with unattached/incorrectly attached kinetochores and plays roles in stabilizing kinetochore–microtubule attachments and in chromosome alignment [9,10]. Together with Bub3, Mad2 and Cdc20, BubR1 forms part of the mitotic checkpoint complex (MCC) that inhibits the anaphase-promoting complex or cyclosome (APC/C) E3 ubiquitin ligase activity towards cyclin B1 and securin. The interaction of SAC kinases Bub1 and BubR1 with the protein Blinkin (also known as KNL1 and Spc105) is required for an efficient mitotic checkpoint response and links SAC signalling with the kinetochore [11,12]. This essential multi-protein complex plays a crucial role in chromosome segregation; it assembles on mitotic or meiotic centromeres and mediates the physical contact of centromeric DNA with microtubules [13,14].

The abundance of DNA breaks in cancer cells containing mitotic spindle abnormalities suggests that mitotic arrest may promote tumorigenesis and antimitotic toxicity as a result of DNA damage [15,16]. The observations that DNA damage induces a delay in the metaphase/anaphase transition, thus leading to defects in kinetochore attachment and function, and that at least one proteinaceous component of the DNA damage response pathway, 53BP1, is localized to the kinetochore during mitosis [17] suggest the possibility of a mechanism of communication between macromolecular assemblies of the DNA repair pathway and the SAC.

2. Non-homologous end joining and spindle assembly checkpoint involve complex multi-component assemblies

Both NHEJ and SAC pathways (figures 1 and 2, respectively) involve the assembly of multi-protein complexes. We begin by briefly describing the state of our knowledge on these systems.
In NHEJ, the association of Ku70 and Ku80 to form a heterodimer is required for double-stranded DNA end-binding activity [18]. In the crystal structure, Ku70 and Ku80 form a pseudosymmetric heterodimer, which encircles duplex DNA [19].
Figure 2. (a) Fluorescence microscopy imaging illustrating the normal and defective segregation of chromosomes. The latter condition triggers the mitotic checkpoint response. Images are found at http://dartmed.dartmouth.edu/summer09/html/discdivision.php. (b) The mitotic checkpoint consists of the protein components Bub1, BubR1, Bub3, Mad2, Cdc20 and Mps1, some of which are recruited to unattached kinetochores when the checkpoint is unsatisfied. Kinetochore localization of Bub1 and BubR1 is mediated by Blinkin, a central component of the kinetochore–microtubule network (KMN). Cytosolic BubR1, Bub3, Mad2 and Cdc20 associate to form the MCC, which has an inhibitory role on the anaphase-promoting complex/cyclosome (APC/C). After bipolar attachment and alignment of all chromosomes at the centre of the cell has occurred, APC/C–Cdc20 inhibition is released by silencing the mitotic checkpoint, thus allowing chromosome separation and mitotic progression from M-phase to interphase. (Online version in colour.)

No contacts with DNA bases and few interactions with the sugar–phosphate backbone are made (figure 3a). No large conformational changes occur in Ku70 and Ku80 on assembling around DNA other than in the C-terminal domains. One DNA face is accessible to processing enzymes that remove damaged nucleotides and fill gaps prior to ligation. These features bring the DNA helix into phase across the junction during end processing and ligation, and provide structural support to broken DNA ends.

Insights into the structures of the DNA-PKcs/Ku70/Ku80 holoenzyme and possible synaptic complexes have been derived from cryo-electron microscopy (EM) and small-angle X-ray scattering (SAXS) studies. Large conformational
changes occur in human DNA-PKcs when double-stranded DNA binds, suggesting that this may correlate with the activation of the kinase [20]. More recently, single-particle electron microscopy studies on human DNA-PKcs/Ku70/Ku80 holoenzyme assembled on DNA at approximately 25 Å resolution [21] have provided further evidence of large conformational changes upon Ku and DNA binding to DNA-PKcs. Moreover, a SAXS study of DNA-PKcs revealed two different modes of dimerization, either head-to-head or palm-to-palm, depending on the presence of either 40 bp hairpin or 40 bp Y-shaped DNA [22].

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Although it has been shown that the interaction between XRCC4 and LigIV is stronger than that of XRCC4 and XLF, it remains to be established whether the XLF dimer interactions with XRCC4 dimer are maintained once the ligase has been recruited. Furthermore, mapping interaction assays have shown that XLF recruitment to DSB ends occurs through interaction with Ku70/80 only in the presence of DNA [23]. XRCC4 is dispensable for XLF recruitment to DNA ends, although it contributes to stabilizing the DNA/XLF complex [23]. Considering that both XLF and XRCC4 require a long piece of DNA for binding, the definition of the structural details of DNA association in the higher order protein complexes should provide new insights into this process.

The three-dimensional structures of binary complexes, which involve direct physical interactions between protein pairs, such as Bub1 and Bub3; Mad3 and Bub3; Mad2–Mad1; Mad2–Cdc20 and BubR1–Blinkin, provide the details of protein–protein interactions essential for SAC signalling. For instance, Bub1 and BubR1 (Mad3 in yeast) have a conserved stretch of about 40 amino acid residues that are predicted to be mainly disordered and to contain the GLE2p-binding sequence (GLEBS) motif, which is identified as the Bub3-binding motif. The crystal structures of two independent complexes formed between yeast Bub3 and peptides that mimic the GLEBS motifs of Mad3 and yeast Bub1 demonstrate that the peptides form an extensive interface along the top surface of Bub3, a single domain protein that shows a canonical WD40-repeat fold organized in seven bladed β-propellers (figure 4a). The interaction is essential for BubR1 kinetochore localization as a single amino acid substitution in the GLEBS motif, and the top face of Bub3 is sufficient to disrupt the interaction, thus leading to extensive defects in chromosome segregation.

Mad1, Mad2 and Cdc20 are other essential components of the SAC signalling pathway. Mad1, a 718 residue coiled-coil protein, the depletion of which severely affects the SAC in mammalian cells [24,25], forms a stable complex with Mad2 in vitro [25]. The crystal structure of Mad2 in complex with Mad1 residues 485–584 (pdb 1GO4) shows that Mad2 exhibits the distinctive HORMA (for Hop1, Rev7 and Mad2) domain, consisting of a single α/β domain organized in three layers: a central layer formed by three α-helices; a large six-stranded β-sheet on the one side; and a short β-hairpin on the other side. In the complex, the Mad1 fragment is predominantly α-helical (figure 4b). Binding studies suggest a conformational mechanism in which Mad1 primes the Mad2 binding site for the interaction with Cdc20 [25]. However, whether Cdc20 and Mad2 can establish direct contacts in vivo remains as a contentious issue.

The crystal structures of the N-terminal regions of yeast Bub1 and human BubR1, which are essential for binding Blinkin, reveal a common fold that comprises a triple-tandem arrangement of the tetratricopeptide repeat (TPR) motif [26,27]. Interactions of Blinkin with TPR Bub1 and TPR BubR1, which are essential for the recruitment of these kinases to the kinetochore, connect SAC signalling with the KMN (KNL1/Mis12/Ndc80) network [11,12,28]. Moreover, depletion of Blinkin in higher organisms by RNAi causes severe chromosomal segregation defects that resemble phenotypes characteristic of Bub1 and BubR1 protein depletion [29,30]. Detailed peptide mapping, physico-chemical, structural and functional analyses support the notion that an N-terminal Blinkin fragment is essential for binding BubR1 and that site-specific substitutions of the latter impair the mitotic checkpoint [12,31].
Figure 4. (a) Structure of Bub3 from *S. cerevisiae* in complex with the GLEB motif of Bub1. (b) Structure of the Mad1–Mad2 tetrameric complex. The crystal structure shows that the two chains of Mad1 interact with Mad2 via the N-terminal coiled-coil region (pdb 1GO4). (c) Crystal structure of the bonsai–Ndc80 complex (pdb 2VE7). Spc24 and Spc25 have N-terminal coiled-coils that mediate inter-subunit interactions, while dimeric Ndc80–Nuf2 comprises N-terminal Calponin homology domains followed by a coiled-coil region engaged in inter-subunit interactions. (Online version in colour.)

3. Weak binary interactions lead cooperatively to well-defined multi-protein complexes

Specific but low-affinity binary complexes leading to cooperative assembly of higher order signalling complexes should be advantageous for signalling [32]. If binary complexes led to signalling in the crowded environment of the cell, opportunistic interactions would likely give rise to noise. On the other hand, multi-protein systems that form cooperatively would less likely form by chance. An example of this phenomenon is the interaction of fibroblast growth factor (FGF) with its receptor FGFR, which plays a role in cell proliferation, differentiation, survival and migration. The structure of fibroblast growth factor
receptor 2 (FGFR2) in complex with its ligand (FGF1) and heparin at 2.8 Å resolution suggests that a 2 : 2 : 1 FGF1–FGFR2–heparin decasaccharide complex may play a role in receptor activation [33]. Heparin is an analogue of the obligate secondary receptor heparan sulphate. Analyses of the complexes with gel filtration, nanospray mass spectrometry and analytical ultracentrifugation [34] demonstrate only a binary 1 : 1 FGF : FGFR complex in the absence of heparin. However, a 2 : 2 : 1 complex forms spontaneously in solution between FGF1, FGFR2 and heparin decasaccharide and less efficiently with octasaccharide. Higher order complexes, e.g. with stoichiometries 4 : 4 : 1, can be observed using mass spectrometry. This probably reflects surface clustering, which is well known to play a role in receptor function [35].

Recently, evidence has emerged for cooperative or synergistic interactions between components of the NHEJ system. Although the interaction between XRCC4 and LigIV leads to a strong 2 : 1 complex, the interactions between XRCC4 and XLF are much more cooperative. Interactions with XRCC4 led to the discovery of XLF [7]. Mutagenesis studies of XLF and XRCC4 suggested that XLF–XRCC4 interactions are mediated through relatively small regions located at the sides of the head domains and contain the helix-turn-helix structures and the β6–β7 loop [36]. SAXS structural studies of XLF–XRCC4 complexes indicated a linear model with approximately a 45° rotation angle between XRCC4 and XLF coiled-coil tails [22]. We have observed an XLF–XRCC4 concentration-dependent higher order complex formation using nano-electrospray ionization mass spectrometry. Furthermore, an 8.5 Å resolution crystal structure of XLF–XRCC4 demonstrates that XLF and XRCC4 dimers interact through their head domains and form an alternating left-handed helical structure with polypeptide coiled-coils and pseudo-dyads of individual XLF and XRCC4 dimers at right angles to the helical axis [37]. In summary, it appears that weak binary interactions between XRCC4 and XLF dimers lead to relatively stable fibres. These are likely to mediate the ligation of DNA in DSB repair through NHEJ.

Cooperative interactions essential for biological complexity also occur in the KMN subcomplexes KNL1, Mis12 and Ndc80. For instance, the crystal structure of the Ndc80 subcomplex shows that it adopts a dumbbell shape (figure 4c) [38–42] containing four subunits: Ndc80 (the subunit that gives its name to the entire subcomplex), Nuf2, Spc24 and Spc25. Two subcomplexes, Spc24–Spc25 and Nuf2–Ndc80, occupy opposite ends of the dumbbell (figure 4c) [38,40]. Cooperative interactions are exemplified by the requirement for the association of Spc24–Spc25 subunits for binding the KNL1 and Mis12 complexes [11,41,42], while the association of the Nuf2–Ndc80 subunits mediates the binding of the Ndc80 complex to microtubules [39,43,44].

4. Low structure complexity and disorder-to-order transitions

The presence of regions of low structure complexity (also referred to as regions of intrinsic local disorder) is widespread in protein molecules [45–52]. They constitute a common feature of hub proteins in interactome networks [53–58]. Indeed, neural network predictors, developed to recognize sequences that correspond to structurally disordered regions, have shown
that 35–51% of eukaryotic proteins have at least one long (i.e. <50 residues) disordered region [59], while DisProt [60], a curated database of protein disorder that provides information about intrinsically disorder proteins (http://www.disprot.org) lists 1375 disorder regions in a total of 643 proteins.

A significant correlation exists between the average predicted disorder per complex and the number of complex components [61]. Intrinsic local disorder can increase the backbone conformational entropy upon ligand binding and provide a kinetic advantage by speeding up the search for specific targets [50,62]. Furthermore, large and highly flexible interaction surfaces can assist the assembly of intertwined multimeric complexes [50]. Indeed, most hubs in protein interaction networks contain long segments of low structural complexity that engage in binding [48,57,63,64] and removal of such hub proteins has a dramatic impact on the function of the entire network [65,66].

A transition from a disorder state to a more organized one may occur upon ligand binding. This process, commonly referred to as a disorder-to-order transition has been described in multiple protein complexes. One example is p27Kip1, the inhibitor of cyclin-dependent kinases (Cdks) and the tumour suppressor p53. Large fragments of p27Kip1 are intrinsically unstructured with a marginal content of helix structure as shown by limited proteolysis, circular dichroism and nuclear magnetic resonance (NMR) spectroscopy analyses [67–69]. The inherent flexibility of unstructured segments is important for sequential p27Kip1 phosphorylation by CK2 (figure 5a) as this post-translational modification primes p27Kip1 for its ubiquitylation and eventual proteolytic degradation, a process required for progression through the cell cycle. Interestingly, a disorder-to-order transition takes place upon p27Kip1 binding to cyclin A–Cdk2 (figure 5b), an interaction that regulates the activity of Cdk2 in a process in which p27Kip1 residues insert into the catalytic cleft mimicking adenosine-S′-triphosphate [70]. A similar disorder-to-order transition has been observed in the interaction of N-terminal p53 with murine double minute (Mdm2) (figure 5c). p53 binding to DM2 results in the loss of transcriptional activity and the stimulation of ubiquitination and degradation by the proteasome. The interaction defines a largely α-helical region and involves extensive van der Waals contacts in which Phe19, Trp23 and Leu26 lying on one side of the helix play a major role [71] (figure 5c). As Mdm2 is often over-expressed in tumours, the Mdm2 interaction with p53 has become an interesting therapeutic target [72,73].

Individual components of the NHEJ- and SAC signalling pathways also fall in this category; they are characterized by the presence of regions of low structural complexity, some of which are disordered prior to assembly and become ordered only on binding. For example, LigIV has a tandem breast cancer gene 1 C-terminal (BRCT) domain with a linker predicted to be mostly disordered. The linker between BRCT1 and BRCT2 mediates the interaction of LigIV with XRCC4 [74,75]. This linker seems to be important for the catalytic activity of LigIV [74]. LigIV asymmetrically interacts with XRCC4 with 1:2 stoichiometry. This transforms the left-handed coiled-coil tail of XRCC4 into the right-handed undecad coil, making the surface of the XRCC4 interaction region flat, as a result of a kink in one of the helices in the coiled-coil (figure 3b,c) [75]. The concerted binding and folding of the previously flexible linker leads to a stable structure in which multiple and well-defined interactions are made between the ligase and the XRCC4.
Figure 5. (a) Far-UV circular dichroism shows that a disorder-to-order transition occurs upon p27Kip1 phosphorylation by CK2 (open circles, p27WT; filled squares, p27-P). (b) p27Kip1 binding to Cdk2–cyclin A and C. A disorder-to-order transitions has been observed in p53 binding to Mdm2. (Online version in colour.)

In SAC signalling, several disorder–order transitions have been observed. For instance, Bub1 and Mad3 GLEBS motifs in complex with Bub3 involve a transition from a predominantly disordered (unbound) to a more ordered (Bub3-bound) state. A similar situation is observed on the interaction between N-terminal Blinkin and BubR1 where Blinkin undergoes an important folding transition upon binding [31]. The disorder-to-order transition opens the possibility that Bub1 and BubR1 binding to N-terminal Blinkin induces
conformational changes that affect the interaction of this kinetochore protein with other ligands such as Aurora B and protein phosphatase 1, thus conferring directionality to the flow of information encoded in this signalling pathway [76].

5. Variation of assemblies over time and in space

The remodelling of macromolecular assemblies in time and space has evolved as a successful strategy that allows sequential obligate steps and the increase of selectivity with a minimal margin for errors. This highly versatile and dynamic nature of remodelling of macromolecular assemblies constitutes a great challenge for their structural characterization, and often requires a multi-disciplinary strategy that combines SAXS, EM, X-ray crystallography, NMR and other biochemical, biophysical, molecular and cellular approaches. In order to understand the temporal organization of the NHEJ repair system as a whole, knowledge of the order of interactions during the assembly of the DNA-PKcs/Ku70/Ku80/DNA ternary complex and the LigIV/XRCC4/XLF/DNA quaternary complex will be essential. The classical sequential model [77–79] suggests that Ku70/80 and DNA-PKcs, which have higher DNA-binding affinity compared with LigIV/XRCC4/XLF, most probably form the DNA-PKcs/Ku70/Ku80/DNA ternary complex first. For the following LigIV/XRCC4/XLF/DNA complex formation, the order and dynamics of protein assembly are still to be determined. The initial interaction between XRCC4 and XLF is relatively weak, but as we have seen earlier, they undergo strong cooperative interactions to form helical structures. Whether these structures are compatible with retention of the XRCC4 interactions with LigIV remains to be determined.

Post-translational modifications (acetylation, phosphorylation, ubiquitylation, etc.) have a significant impact on protein function as they can affect protein stability, turnover, reversibility, sub-cellular localization and/or the hierarchical order of assembly/disassembly [80,81]. For instance, in NHEJ signalling, phosphorylation of DNA-PKcs induces a large conformational change, sufficient to open the gap in the ring and provide access to or release from DNA [82]. The C-terminal domain of Ku80 (Ku80CTD) has been shown to be flexible and to extend in solution to the benefit of recruitment of DNA-PKcs, suggesting that the interacting of Ku80 with DNA-PKcs occurs on both sides of DSBs [22]. Although phosphorylation of LigIV, XRCC4 and XLF by DNA-PKcs does not interfere greatly with the core functions of these proteins, their differential phosphorylation probably regulates the correct spatial arrangement of the higher order complexes by altering the relative binding affinities of various protein–protein or protein–DNA interactions. Further studies should aim to characterize these complexes temporally as well as spatially.

Some experimental studies have suggested a two-phase NHEJ protein-binding model [23]. This has been investigated by Li & Cucinotta [83], who have proposed a mathematical model describing the biochemical reaction network. They suggest that, if a DSB repair pathway independent of DNA-PKcs exists, then the sequential model is indistinguishable from the two-phase model where after binding of Ku70/Ku80 with DNA, the other NHEJ components can all be recruited to DNA sites and then rearrange and change interaction modes according to the process required.

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Knowledge of the recruitment hierarchy among the kinetochore subunits should eventually provide clues about the mechanisms mediating kinetochore function and its regulation by the SAC. However, the specific roles of a large number of proteins that participate in the assembly and regulation of the kinetochore remain to be established. For instance, Ohta and collaborators have combined stable-isotope labelling by amino acids in cell culture mass spectrometric techniques with bioinformatics analyses to identify chromosomal proteins in the context of intact chromosomes [84]. This large-scale quantitative mass spectrometry study allowed the identification of 4029 mitotic chromosome-associated proteins, of which 562 were previously uncharacterized; there is clearly much remaining of the complexity of these systems to define.

An important consideration in SAC signalling is that the link made by the KMN network to connect the centromere to the microtubule fibre of the mitotic spindle must be strong enough to sustain the pulling forces during anaphase. At the same time, it must be sufficiently dynamic to permit the polymerization–depolymerization of the plus ends of microtubules and respond efficiently to the regulatory mechanisms that enable chromosomes to align at the metaphase plate prior to anaphase. Thus, a number of properties are required to ensure the flow of information in order to signal both correct and incorrect attachment of chromosomes, to enable a weakening of microtubule binding when there is a need to correct the attachment, and to delay anaphase until proper attachment has occurred.

The mode of assembly and regulation of macromolecular complexes of NHEJ and SAC checkpoint signalling pathways are reminiscent of Mackay’s early ideas on the physical meaning of hierarchy in complex structures [85], which recalls Bernal’s definition of life as ‘a partial, continuous, progressive, multi-form and conditionally interactive, self-realization of the potentialities of atomic electron states’ [86]. As the use of high-throughput experimental techniques, together with molecular and computational methods of systems biology, begin to give insights into the dynamic architecture of the interactome, we will learn more of the exquisite complexity of interactions between thousands of protein components participating in finely tuned regulatory pathways essential to all eukaryotes. Definition of genotype–phenotype associations at different levels of complexity, i.e. genes, RNA transcripts, peptides, proteins, metabolites and environmental factors, that characterize cellular networks can only be understood in the light of evolution and should expose the mechanisms and principles underlying the regulation of complex biological systems [87–89].

6. Closing remarks

The functions of eukaryotic cells depend upon the organization of macromolecular assemblies. Such organization is a structural theme that occurs widely in the regulation of signalling pathways. Regions of low structural complexity play essential roles in this process as they allow greater selectivity in molecular recognition and will tend to increase signal-to-noise ratio. Further selectivity is gained by the involvement of multiple components in systems regulating DNA DSB repair by NHEJ recombination and SAC signalling. A spatial and temporal understanding of NHEJ and SAC signalling should provide insights into the mechanism of these critical cellular processes.
The combination of structural biology of macromolecular assemblies, together with systems biology approaches, should lead to a more holistic understanding of the cell and provide insights into how defects in molecular interactions can lead to cellular misfunction. Databases that enable the utilization of the available structural information on protein interactions with small molecules in combination with sequence and molecular interaction data [90,91] are likely to be central to the design of useful chemical tools for dissecting these complex pathways and for providing a basis for drug discovery in the coming years.

In this review, we have demonstrated the importance of crystal structures for the derivation of knowledge of macromolecular interactions and architecture, but we have also shown how we can go ‘beyond crystals’ in order to infer molecular and cellular function in space and time. We have observed how information and structure interact: information defines structure but material structure and organization in turn define information.

References


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