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Challenges of studying 14-3-3 protein-protein interactions with full-length protein partners

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The formation of protein-complexes, or so-called protein-protein interactions (PPIs), is crucial for many physiological and pathological processes. The human interactome is estimated to contain around 130,000–650,000 different PPIs (1). Therefore, it is critical to obtain a detailed understanding of the structural characteristics and the mechanism of action of individual PPIs to acquire extended knowledge on cellular processes. Additionally, PPIs have become popular drug targets in the last two decades, as they greatly expand the druggable genome beyond classical drug targets like enzymes and receptors (1,2). This further encouraged the identification of PPIs and their role in pathological processes.

A crucial role within the protein interactome can be found for the hub-protein 14-3-3. This protein is known to bind hundreds of different, mainly phosphorylated, protein partners. Upon binding of its protein partners, 14-3-3 can modulate their stability, activity, and/or localization. Among its binding partners are many well-known drug targets such as Raf kinases, the tumor suppressor p53, and the estrogen receptor. This makes 14-3-3 heavily involved in many pathological processes, which raised significant interest in 14-3-3 PPIs as drug discovery targets (3).

Despite the interest in using 14-3-3 PPIs as a drug target, *in vitro* studies on these PPIs often remain challenging (Fig. 1 *a*). Only in very few cases was structural data obtained of 14-3-3 bound to a full-length protein partner, like most recently done for the B-Raf/14-3-3 PPI (4,5) (Fig. 1 *b*). However, frequently, the phosphorylated residue binding in the 14-3-3 binding groove is located within a disordered region, or the binding partner is, in general, an intrinsically disordered protein. Because of this disordered nature, it is challenging to study the structure of these PPIs using classical techniques such as x-ray crystallography and cryoelectron microscopy (cryo-EM). Therefore, the 14-3-3 binding motif is, in many studies, represented by a synthetic phosphopeptide, making it easier to determine the structural characteristics and the binding affinities between 14-3-3 and its binding partner (3). However, by using this simplified peptide mimic, much structural and mechanistic information might be lost between 14-3-3 and the protein partner, highlighting the importance of studying 14-3-3 PPIs using both full-length partners.

To acquire structural and mechanistic understanding of 14-3-3 PPIs for which it is difficult to obtain a crystal or cryo-EM structure, alternative techniques are needed. The groups of Veronika Obsilova and Tomas Obsil are world-leading experts in studying these challenging 14-3-3 PPIs. They have used a wide range of biophysical techniques, such as small-angle x-ray scattering, chemical crosslinking, and analytical ultracentrifugation, to characterize 14-3-3 interactions with, among other partners, DAPK2, CaMKK2, and caspase-2 (6–8) (Fig. 1 *c*). Thereby, they were able to obtain structural and mechanistic understanding of these PPIs without the need for x-ray crystallography and cryo-EM structures.

This issue of *Biophysical Journal* contains a recent study by Joshi et al. that describes the interaction between 14-3-3 and the E3 ubiquitin ligase neural precursor cell expressed developmentally down-regulated 4-ligase (Nedd4-2) (9). They provide a systematic way to investigate the influence of 14-3-3 binding on the accessibility and mobility of individual Nedd4-2 domains using fluorescence-based methods. With this study, the group expanded the toolbox of biophysical techniques to characterize 14-3-3 PPIs (Fig. 1 *c*). This work is a follow-up story on earlier research by this group where they identified the phosphorylation sites that are crucial for 14-3-3 binding and in which initial crosslinking and SAXS experiments indicated that 14-3-3 binding induces

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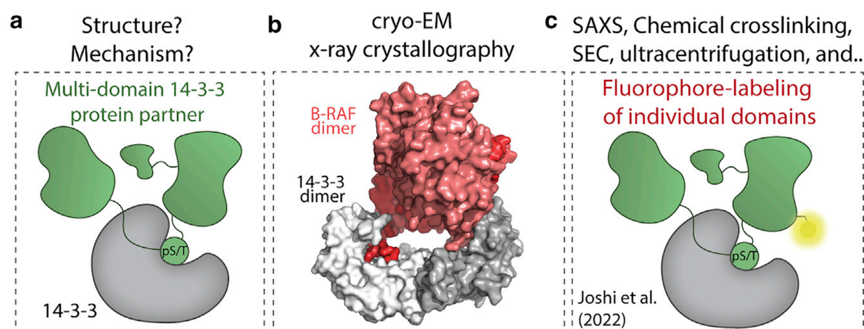

FIGURE 1 Methods to study 14-3-3/partner protein complexes. (*a*) Schematic representation of multi-domain phosphorylated protein (green) binding to 14-3-3 (gray), raising the question of structure and mechanism of action. (*b*) First-line techniques to look at mechanism and structure are cryo-EM and x-ray crystallography. Example of crystal structure of 14-3-3 (gray) with BRAF (red) (PDB: 6U2H). (*c*) Alternatives to study PPIs in the absence of structural data including fluorophore labeling of individual domains as used by Joshi et al. To see this figure in color, go online.

structural rearrangements of the Nedd4-2 protein (10). These results encouraged the research group to further study the influence of 14-3-3 binding on Nedd4-2 functioning.

The Nedd4-2 protein contains four WW domains, which play a crucial role in binding proline-rich (PPXY) motifs in its substrates. Furthermore, it contains a catalytic HECT domain for poly-ubiquitination of its substrates. High-affinity 14-3-3 binding to Nedd4-2 is observed upon phosphorylation of serines 342 and 448 of Nedd4-2. These phosphorylation sites are present in disordered regions of the Nedd4-2 protein and surround the WW2 domain of Nedd4-2. While previous work was able to identify these phosphorylation sites, the actual mechanism of action upon 14-3-3 binding remained unknown.

To determine the effect of 14-3-3 binding on the individual WW and HECT domains of the Nedd4-2 protein, Joshi et al. designed eight different Nedd4-2 constructs that were site-specifically labeled with the polarity-sensitive probe AEDANS. In each of these constructs, the probe was strategically installed in proximity to one of the WW domains or the HECT domain. These constructs then allowed a systematic study on the effect of 14-3-3 binding to Nedd4-2 regarding the accessibility and mobility of each individual domain. Measurements of the probe lifetime, anisotropy decay, and fluorescence quenching were performed of each of the probe-functionalized Nedd4-2 proteins in the absence and presence of 14-3-3. A change in these three physical properties of the probe upon the addition of 14-3-3 indicated that 14-3-3 binding influences the domain that was functionalized with the AEDANS probe.

The fluorescence-based experiments showed that 14-3-3 is able to induce structural rearrangement of Nedd4-2. Mainly the WW3 and WW4 domain were influenced by the addition of 14-3-3, in which a reduction of domain mobility was observed. Similar effects were detected for WW2, although to lesser extent. The most N-terminal WW1, on the other hand, did not seem to be influenced upon 14-3-3 binding. An opposite effect was noticed for the catalytic HECT domain, which became more mobile and solvent exposed upon 14-3-3 binding. Together with the trypsin digestion studies, showing reduction of WW3, WW4, and HECT proteolytic degradation in presence of 14-3-3, this work demonstrated how 14-3-3 influences individual domains of the Nedd4-2 protein, thereby providing valuable information on the mechanism behind 14-3-3 modulation of Nedd4-2 activity.

In summary, the current work by Joshi et al. provides a systematic way to identify the influence of 14-3-3 binding to Nedd4-2, which easily can be translated to other PPIs. They gained a large amount of information from the same probe-functionalized construct by measuring several physical properties of the probe. This not only provided information on the structural characteristics of this PPI but also allowed their group to get a mechanistic understanding of it, which is even challenging to determine with established techniques such as x-ray crystallography and cryo-EM. Therefore, this work by Joshi et al., together with their earlier published work regarding 14-3-3 full-length PPIs, is extremely valuable to get more insight in the binding mechanisms 14-3-3 PPIs and the identification of 14-3-3 PPIs as drug targets.

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