Structural Reconstruction of Protein-Protein Complexes Involved in Intracellular Signaling

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Abstract

Signaling complexes within the cell convert extracellular cues into physiological outcomes. Their assembly involves signaling enzymes, allosteric regulators and scaffold proteins that often contain long stretches of disordered protein regions, display multi-domain architectures, and binding affinity between individual components is low. These features are indispensable for their central roles as dynamic information processing hubs, on the other hand they also make reconstruction of structurally homogeneous complex samples highly challenging. In this present chapter we discuss protein machinery which influences extracellular signal reception, intracellular pathway activity, and cytoskeletal or transcriptional activity.

Keywords

Cellular signaling • X-ray crystallography • Cryo-EM • Protein-protein interactions • Signaling pathway

20.1 Introduction

Signal transduction refers to all molecular events between the reception of extracellular signals and the mounting of biologically appropriate responses inside the cell (*e.g.*, gene expression by the general transcriptional machinery or movements involving cytoskeletal proteins). As cells receive myriad of signals and responses are functionally diverse, a great proportion of intracellular proteins participate in the hierarchical assembly of signaling complexes. Protein-protein interaction specificity of components within these complexes determines how signaling pathways are wired. We show that detailed mechanistic understanding on how signaling complexes transmit intracellular information requires their structural reconstruction. However, this is difficult, because signaling proteins often form short-lived transient complexes, are prone to allosteric regulatory mechanisms, and modulated by post-translational modifications (*e.g.*, phosphorylation

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or nondegradative ubiquitinilation). In addition, most proteins contain long disordered protein regions, display multi-domain architecture, and binding affinities between structured and linear motif containing disordered regions are weak (micromolar).

In the next pages we will review how the above mentioned technical challenges were solved for reconstructing GPCR-G protein complexes, focal adhesion sites, the Ste5 MAP kinase cascade, the ARP2/3, and the Mediator complex. The structural reconstruction of these complexes has given insight into the reception of chemical ligands, adhesion to the extracellular matrix, intracellular signaling cascade insulation, actin branching dynamics and transcriptional activation, respectively. These topics give a cross-section of now structurally explored molecular events from the cellular signaling field. On the other hand, the examples below maybe viewed as paradigmatic cases on how to devise strategies to limit conformational flexibility of reconstituted multi-protein complexes, or alternatively to divide them up into functionally relevant and structurally compact units.

20.2 Sensing the Chemical Environment: GPCRs and Heterotrimeric G Proteins

G protein-coupled receptors (GPCRs) play a central role in detecting extracellular signals. They bind ligands outside of the cell, go through binding triggered conformation changes and turn these into downstream intracellular signals with the help of heterotrimeric G-proteins located at the cytoplasmic side of the cell membrane. GPCRs are an important group of signaling receptors as the largest part of current drugs deliver their effects through them. Learning the molecular mechanism of GPCR activation is the key to create successful therapeutics. However, acquiring insight into the conformational changes of GPCRs upon ligand binding has turned out to be a difficult task [1]. The first solved GPCR structure was the light sensitive but relatively stable rhodopsin [2]. Most GPCR proteins are hard to express in the necessary amounts and are unstable when using common detergent solubilization methods. Instead of detergents, most of the GPCR crystals were grown in lipidic cubic phase where proteins are stabilized by the membrane bilayer [3].

The highly dynamic nature of GPCRs has hampered their structural investigation for a long time. Structure solution of the beta-2-adrenergic receptor (β2AR) was made possible by using monoclonal antibodies to stabilize conformation of a flexible region—the third intracellular loop connecting two transmembrane regions, helices 5 and 6 [4]. As an alternative to this, insertion of the stable T4 lysozyme (T4L) protein at this region was also successfully used to stabilize the GPCR structure [5]. In addition, using inverse agonists such as carazolol was helpful in locking the GPCR into its inactive conformation, causing less conformational heterogeneity at other flexible protein regions [6].

Obtaining an active, agonist-bound GPCR structure has also proven difficult due to the inherent instability of this state in the absence of a G protein. This was circumvented by using nanobodies, single domain antibodies that exhibited G protein-like behaviour [7, 8].

Binding of agonists to the extracellular region of the GPCR induces a conformational change in the receptor. The activated GPCR receptor allosterically activates the heterotrimeric G protein. The activated G proteins alpha subunit (Gα) exchanges GDP for GTP, which results in the dissociation of the Gα from the Gβ-Gγ subunits. Activated Gs protein binds and then turns on adenyl cyclase (Fig. 20.1). In 2011 the β2AR-Gs protein complex was finally solved [8]. This was a great contribution to fully understanding the molecular mechanism behind GPCR signaling as well as to know how most drugs exercise their effect. Similarly to efforts on the monomeric GPCR, fighting against and prevailing over conformational heterogeneity of receptor samples was the key for success and several former methodological improvements on how to handle GPCR samples had to be combined. The fusion of the T4L protein as well as the use of a nanobody

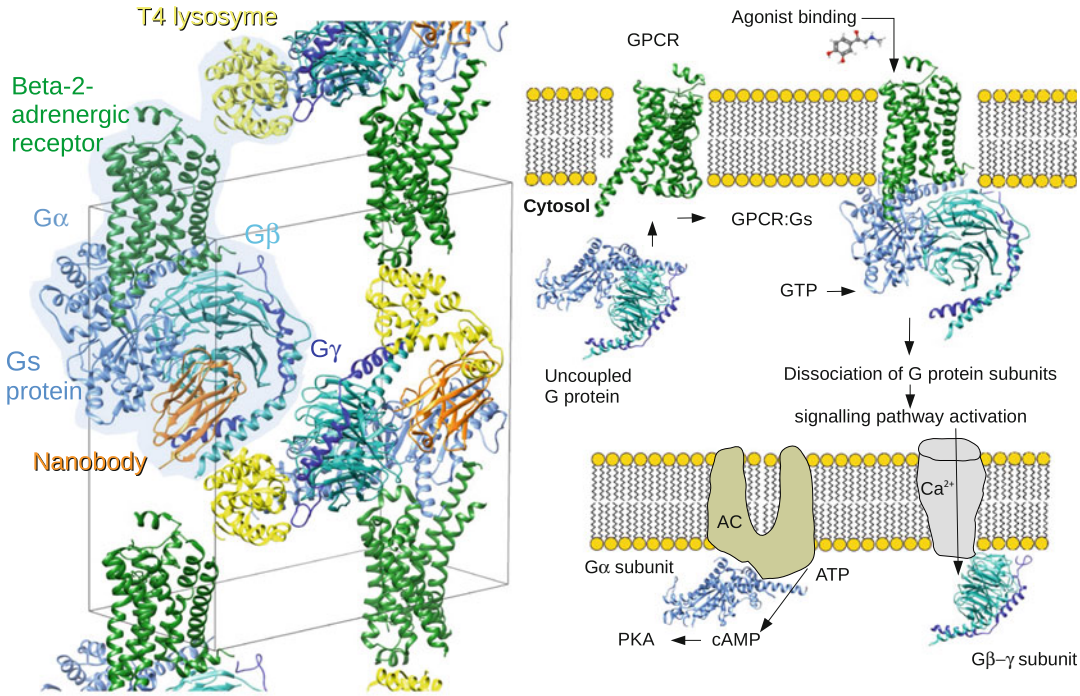

Fig. 20.1 GPCR structure and signaling. The panel on the *left* displays the unit cell of the crystal structure of the beta-2-adrenergic receptor and the Gs protein complex. The T4L fusion contributes to the crystal lattice contacts and the nanobody stabilizes a signaling-competent conformation of Gs. One T4L-β2AR-Gs-Nb35 nanobody complex is highlighted in *blue* background. Panels on the *right* show the signaling events after GPCR ligand binding. The activated Gs protein dissociates, its alpha subunit activates adenyl cyclase (AC) and the produced cAMP activates Protein kinase A [49, 50]. The G protein beta and gamma subunit complex also have regulatory functions. It is known to have regulatory effect on calcium ion channels for example [51]

(Nb35) was necessary to stabilize the complex and provide optimal crystal lattice contacts. Finally the T4L-β2AR-Gs-Nb35 protein complex was successfully crystallized in lipidic cubic phase (Fig. 20.1).

Understanding how ligands induce activating conformational changes in GPCRs required some truly creative and novel methods to be applied for their crystallization. Many years of method developments were required to learn how it is pragmatically possible to decrease the inherent flexibility of these dynamic molecular switches.

20.3 Sensing the Matrix: Focal Adhesions

The focal adhesion of cells to the extracellular matrix (ECM) or to neighboring cells is an interesting example for showing how cells could gain information about their physical environment. Focal adhesions are macromolecular assemblies connecting cells to physical surfaces. In adhesion signaling the recruitment of many adaptor proteins to the plasma membrane mediate the outcome of the response. Integrins have a major role in forming focal adhesions and in transducing biochemical signals. Major components of "integrin adhesomes" are paxillin, talin, and vinculin. Overall, they may be composed of more than 150 components and closer examination of this complex network revealed the existence of functional subnets. Key network motifs were dominated by three-component complexes in which a scaffolding molecule recruits both a signaling molecule and its downstream target [9]. Integrin signaling plays a role in cell migration, immune and inflammatory responses, and also in actin polymerization involving the ARP2/3 complex (see later).

The characterization of protein-protein interactions in adhesion contacts are mostly based on Fluorescence Resonance Energy Transfer (FRET), fluorescence co-localization, acceptor photobleaching FRET (apFRET), Fluorescence Recovery After Photobleaching (FRAP) based assays and immunofluorescence imaging [10–12]. These studies revealed interacting proteins at focal adhesion sites. Since many structures of key protein-protein complexes are not known, most molecular mechanisms still remain undiscovered.

Cryo-electron tomography recently gave fundamental insight into the core of focal adhesion sites within cells [13]. Under cryogenic conditions focal adhesions were identified in cells by fluorescent microscopy based on YFP labeled paxillin and by immunolabelled vinculin. The identified components were indexed for cryo-electron tomography. As a result it was possible to identify adhesion related intact integrin-paxilin-vinculin-actin complexes, and their structure could be revealed at ~4–6 nm resolution. As complexes were analyzed in cells, imaging gave information about localization of adhesome particles within the cell. This analysis revealed that the membrane–cytoskeleton interaction at focal adhesions is indeed mediated through particles that are directly attached to actin fibers (Fig. 20.2).

Integrins are heterodimeric receptors of alpha and beta subunits and they are linked to the intracellular cytoskeleton through their short cytoplasmic tails [14, 15]. These cytoplasmic tails are flexible and serve as a hub for adaptor proteins that recruit other interaction partners [16]. Paxillin is one of the well-characterized adaptor protein for integrins which integrates signaling and structural proteins into adhesion sites. It functions as a platform to coordinate multiple signaling pathways and to control the reorganization of the cytoskeleton. One of its major partner is focal adhesion kinase (FAK) which is a central signaling protein recruited to adhesomes. FAK is a multi-domain tyrosine kinase [17]. NMR studies on the interacting domains of FAK (FAT domain) and paxillin (LD motifs) revealed the highly dynamic nature of this important regulatory interaction [18]. Focal adhesions are abundant in regulatory proteins such as protein kinases, phosphatases, GTPases, GAPs, and GEFs. Because these are not only affected by upstream signaling events coming from the receptor but in turn they also modify the receptor, integrin signaling is a two-way signaling process where besides mediating signals from outside to inside, cells could alter their integrin binding affinity to its ligands for inside-out signaling [19] (Fig. 20.2).

Focal adhesions are complex and dynamic structures comprised of high number of protein components. Once protein binding profiles are mapped out, structural investigation of important binary or ternary sub-complexes is possible, however, understanding how they connect integrin receptors to the cytoskeleton will naturally require investigation of at least the core complex in the cell. Cryo-electron tomography on specifically labeled multi-protein containing cellular structures gives unique structural information, albeit at low resolution, which is not possible through reconstituting complexes from purified components *in vitro*.

20.4 Organizing Protein Kinases into Functional Modules

Intracellular signaling pathways often use cascades of protein kinases to mediate signals from the cell membrane. Interestingly, signaling cascades often use shared enzymatic components. At the mechanistic level the question then arises as to how functionally distinct pathway activities are insulated. The solution may be the use of multi-domain scaffolds consisting of dedicated binding proteins capable of assembling different sets of protein kinases. Scaffold proteins potentially allow the combinatorial use of a limited set of signaling enzymes to control a great number of signaling activities [20]. Scaffolds, however, do not merely facilitate signaling between recruited enzymatic components by passive tethering but they also allosterically modulate their bound partners. Recent studies on scaffolds of mitogen activated protein kinase (MAPK) pathways

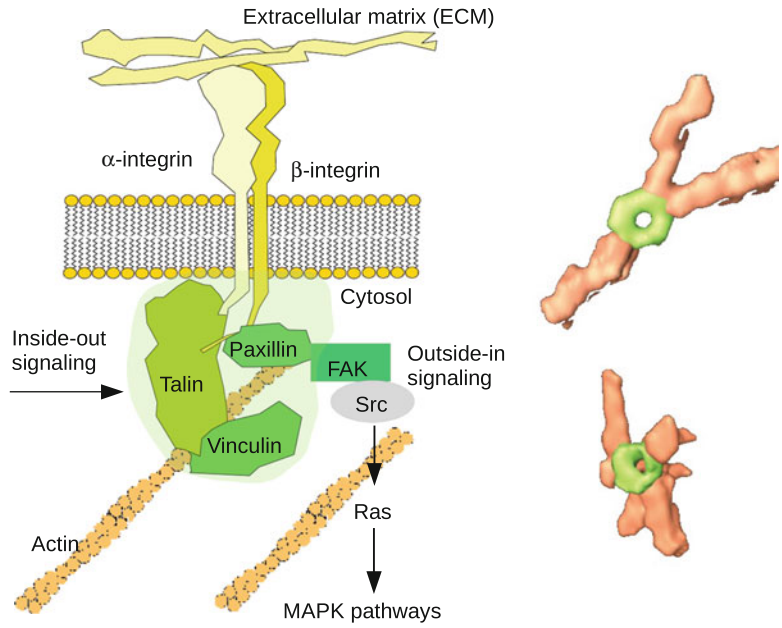

Fig. 20.2 Focal adhesion sites. Schematic representation of adhesome particles. The complex shown in green contains the paxilin-vinculin-talin adapter complex that couples integrin receptors to actin and to the focal adhesion kinase (FAK). The recruitment of kinases (*e.g.*, FAK and Src) ensures the functional linkage to downstream signaling pathways (*e.g.,* Ras/MAPK). Besides this outside-in signaling, integrin can be regulated through talin by inside-out signaling. Panels on the *right* show two different adhesome relevant particles comprised of paxilin, talin and vinculin (in *green*) connected to the cytoskeleton (actin in salmon) (Cryo-electron tomography images were taken from Ref. [13])

demonstrated this elegantly by reconstituting scaffolded MAPK modules out of components in well-defined conformational states [21].

One of the best characterized signaling pathway is the baker's yeast α-pheromone response (mating pathway) [22]. This is a classical GPCR triggered pathway that is dependent on an evolutionarily conserved, three-tiered kinase cascade (Fig. 20.3). The three kinases (Ste11, Ste7 and Fus3) sequentially activate each other and can simultaneously bind to the Ste5 scaffold protein. Upon activation of the GPCR the dissociated βγ-subunit of the G-protein recruits Ste5 to the cell membrane, which brings about the activation of the first protein kinase, Ste11, by a membrane located kinase, Ste20. In turn, Ste7 gets activated which will then activate the Fus3 mitogen-activated protein kinase (MAPK). Activated Fus3 enters the nucleus and phosphorylate transcription factors that execute the mating response (where a-type haploid cells fuse with α-type haploid cells to form diploids.) Interestingly, other physiologically non-related pathways also use Ste7 as a common signaling mediator. For example the filamentous growth pathway depends on the Ste7 mediated activation of the Kss1 MAPK. How can Fus3 be selectively activated by Ste7 molecules that obtained upstream signals from the mating but not from the filamentous growth pathway? The answer lies in the Ste5 dependent allosteric activation mechanism of Fus3 by Ste7. In contrast to Kss1, Fus3 can only be activated by Ste7 if it is co-bound with its activator kinase on the Ste5 scaffold [23]. In addition, Ste5 itself is also allosterically regulated. An internal interaction between two of its domains hinders its allosteric role on Ste7-Fus3 signaling, while this is relieved upon its membrane recruitment following GPCR activation [24]. These mechanisms ensure that Ste7 can be used in two unrelated pathways in a physiologically relevant fashion.

Scaffold proteins are abundantly used in MAPK signaling pathways [25]. Similar recon-

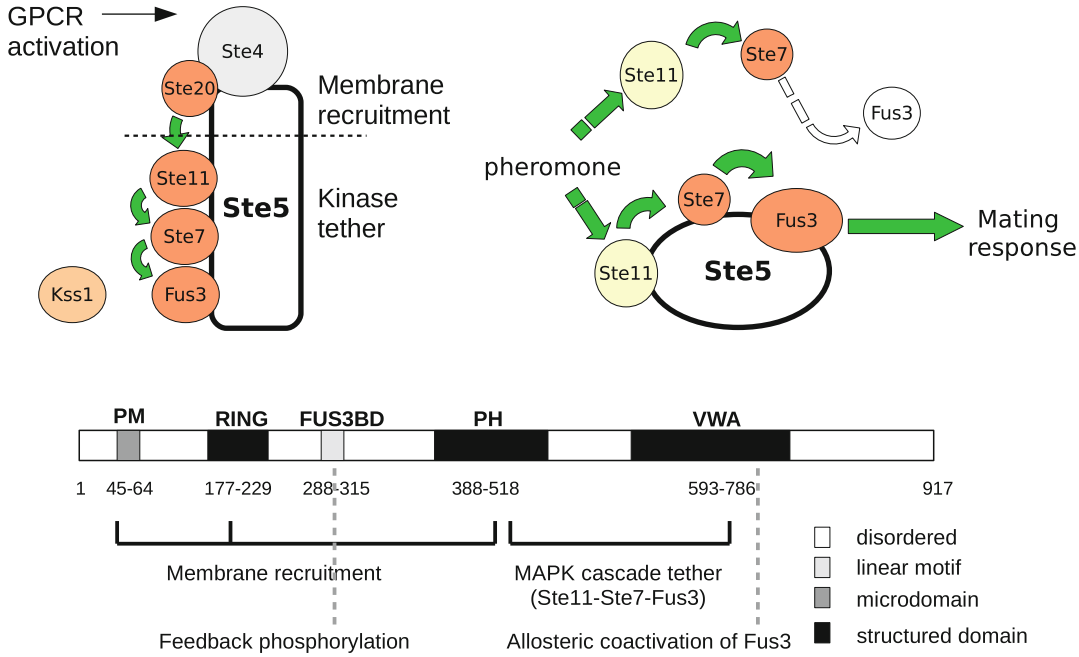

Fig. 20.3 Modular interactions of the Ste5 scaffold. Ste5 contains close to 1,000 amino acids. Long stretches of disordered protein regions are interspersed with differently structured regions. PM is an amphipathic alpha helix that binds membranes, the RING domain binds to Ste4 which is the β-subunit of the heterotrimeric G-protein, the Fus3 binding domain (FUS3BD) is a linear motif that adopts a defined conformation only when it is bound to the Fus3 kinase, the pleckstrin homology (PH) domain binds to membrane phosphoinositides, and von Willebrand type A (VWA) domain binds Ste7. Some of these regions play a role in the core steps of signal propagation through the scaffolded complex (*e.g.*, membrane recruitment, tethering MAPK cascade components and allosteric coactivation of the MAPK), while others are involved in higher-order regulatory mechanisms (*e.g.*, negative regulation of membrane recruitment by other kinases, PM; or feed-back phosphorylation by the activated MAPK, FUS3BD)

stitution studies as described above with MAPK module components of the epidermal growth factor sensing pathway also highlighted the importance of allosteric regulation and the existence of multiple, dynamic conformational states. This pathway culminates in the activation of the mammalian MAPK homolog of Fus3, ERK2, and it contains the three-tiered Raf-MEK-ERK module where the KSR scaffold plays somewhat analogous functions to that of Ste5. Here structural studies on sub-complexes of this module showed that KSR-Raf heterodimerization results in an increase of Raf-induced MEK phosphorylation via the KSR-mediated relay of a signal from Raf to release the activation segment of MEK for phosphorylation [26].

Scaffold proteins are normally multi-domain proteins comprised of folded domains and linear motifs with long stretches of disordered protein regions linking these together. Their bound enzymatic components and even scaffolds themselves are subject to function modifying modifications as well as to mutual allosteric regulation. These make the reconstitution of complete scaffolded modules in well-defined functional states technically impossible. The main problem is that these complexes even if reconstituted from homogenous protein sample components, they are too flexible, and thus too heterogeneous for any single particle cryo-EM or crystallography based approaches, and far too big for NMR. Thus researchers have used the "divide and conquer" strategy and focused on characterizing the nature of binary interactions between scaffold-kinase and kinase-kinase pairs. The mechanistic understanding on how the functionally meaningful scaffolded module works comes from by piecing together data obtained on sub-complexes.

20.5 Controlling Cytoskeletal Structure and Dynamics

The dynamic polymerization, depolymerization and branching of actin filaments are controlled by more than a hundred actin-binding proteins [27]. How upstream signals influence this complex network? One of the most studied regulator complex is the actin-related protein-2/3 (ARP2/3) complex, which is responsible for the formation of branched actin filaments. Structural reconstitution experiments seek to reveal the regulatory mechanism of this complex in order to better understand its role in various processes from cell migration, endocytosis, vesicle trafficking, cytokinesis to tumor-cell invasion and metastasis [28]. ARP2/3 is a stable complex of seven conserved subunits (Fig. 20.4). ARPC2 and ARPC4 form the structural core of the complex, ARP2 and ARP3 are involved in the nucleation process, and ARPC1, ARPC3, and ARPC5 contribute to the activation of the complex by N-WASP (neuronal Wiskott-Aldrich Syndrome Protein). Upstream activators responsible for actin regulation (*e.g.*, Cdc42-GTP and PIP2) can bind N-WASP, which disrupts its auto-inhibitory intramolecular interaction. The unmasked VCA domain can bind ARP2 and ARP3 subunits, and branching will be started by binding to the mother actin filament. The pseudo actin dimer composed of ARP2 and ARP3 act as a template for the building of the new filament joining to its mother with 70° Y angle [29, 30].

The reconstitution of human recombinant ARP2/3 complex provided insights into the role of the individual subunits on the stability of the complex as well as on the nucleation of branched filaments [31]. During the reconstitution of any complex it is necessary to fix the conformational states of the monomers to gain a homogeneous sample. In the case of transient interactions it is particularly challenging to determine the conditions for capturing the complex in its active state. For the ARP2/3 complex, several studies tried to resolve the inactive and active states. Beyond crystallization—which has the limitation of freezing the complex into only one state—cryo-EM has been applied to follow transitions between different molecular states [30]. *In vitro* reconstitution of the active ARP2/3 complex is a multi-step process. The active conformation is the result of a conformational change that brings ARP2 and ARP3 subunits together mimicking two sequential subunits in an actin filament (Fig. 20.4). This process requires many components: ATP, Mg, N-WASP, mother actin filament and G-actin monomers.

The first solved crystal structure was the bovine ARP2/3 complex in its inactive state [32]. The architecture confirmed the structural similarities of ARP2 and ARP3 with actin as well as the central role of the core proteins ARPC2 and ARPC4. Homology modeling showed that the important contact points and residues are evolutionary all conserved [33]. *In vitro* FRET studies proved that binding both the nucleotide and NPF is essential for the formation of active ARP2/3 complex [34]. YFP or GFP labeling of individual subunits of the complex enabled their docking into electron micrographs obtained on reconstituted branched actin [35].

Three conformational classes of particles were discovered on the EM grids of wild type yeast and bovine ARP2/3 samples [36]. The open, intermediate and closed states imply great structural flexibility. Further examination revealed that the cryo-EM maps changed when the complex bound to regulator molecules: the inhibitor coronin bound to the ARPC2 subunit and stabilized the open complex, while the activator N-WASP locked it into the closed (active) conformation.

Complex regulatory machines may exist in multiple conformational states and structural reconstitutions first should target only the core part responsible for setting up the basic architecture of the complex. Later, including components outside the core is not only necessary to mechanistically understand activation but also to stabilize conformations that represent important functional states.

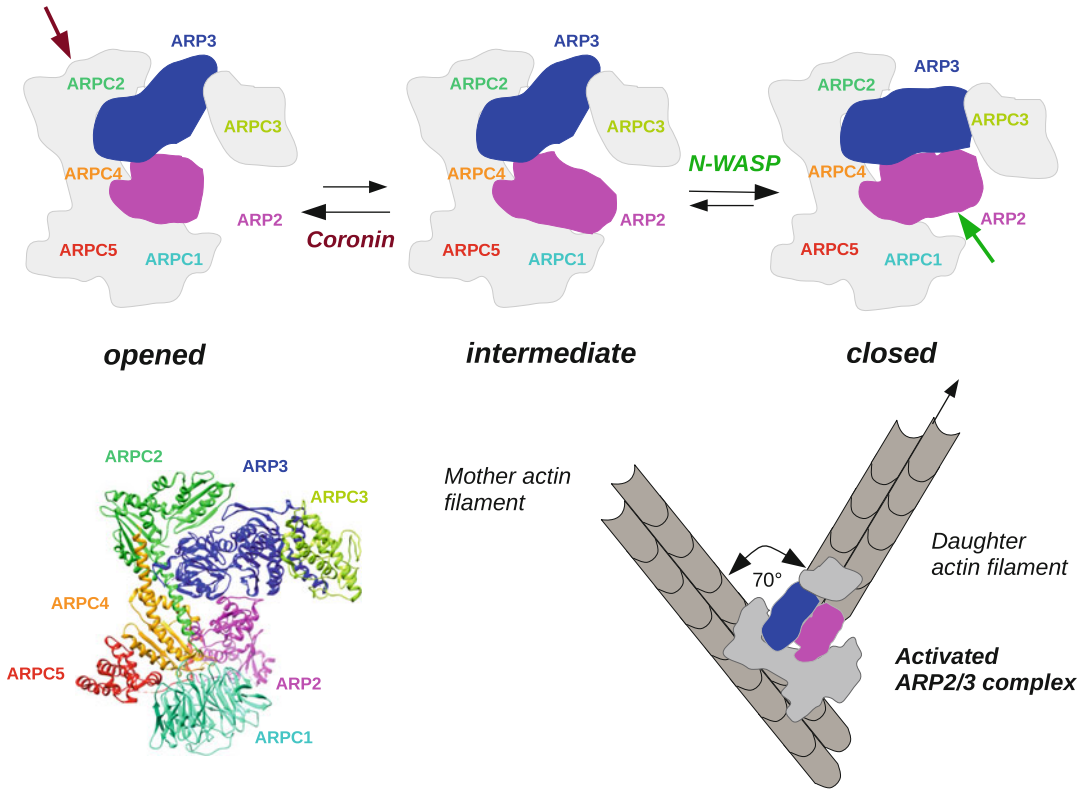

Fig. 20.4 Structural alteration steps leading to active ARP2/3 complex. Model of structural transition between inactive (opened), intermediate and active (closed) ARP2/3 complex observed in cryo-EM structural reconstructions. Terminal stages can be stabilized by inhibitor (coronin) or activator (N-WASP) proteins (their binding site is shown with *arrows*). Crystal structure of inactive ARP2/3 (PDB ID: 2P9L) fits the model of the opened state [36]. The figure on the right shows the branched actin filament bound by the ARP2/3 complex in its closed state. ARP2 and ARP3 (*magenta* and *blue*, respectively) mimic two actin monomers in the closed state of the ARP2/3 complex, thus they act as a template for the new filament growing in approximately 70° compared to the mother filament

20.6 Protein Complexes Controlling Transcription

A signaling pathway most often influences the transcription of selected genes. To understand transcription regulation, researchers in the last decade have reconstituted core transcriptional complexes [37]. These studies highlighted the importance of transient structural changes forming in response to activator or repressor molecules. The most studied complex is the class II transcription pre-initiation complex (PIC), which is a 4 MDa multi-protein assembly comprised of 60 polypeptides. PIC is comprised of RNA polymerase II (Pol II), general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH), and the Mediator complex [38] (Fig. 20.5). There are crystal structures available for some of the individual proteins and these could be used for docking them into the cryo-EM maps obtained on larger assemblies [39].

The 26-subunit Mediator complex acts like a bridge for signal transduction between transcription factors and RNA polymerase II. Its large surface area enables it to accept multiple inputs from transcription factors, co-activators, co-repressors, or nucleic acids. A simple input signal may be for example the appearance of an activated transcription factor on the DNA enhancer. According to the multiple allosteric network model, the input signal causes binding factor specific structural shifts which spreads across the whole complex

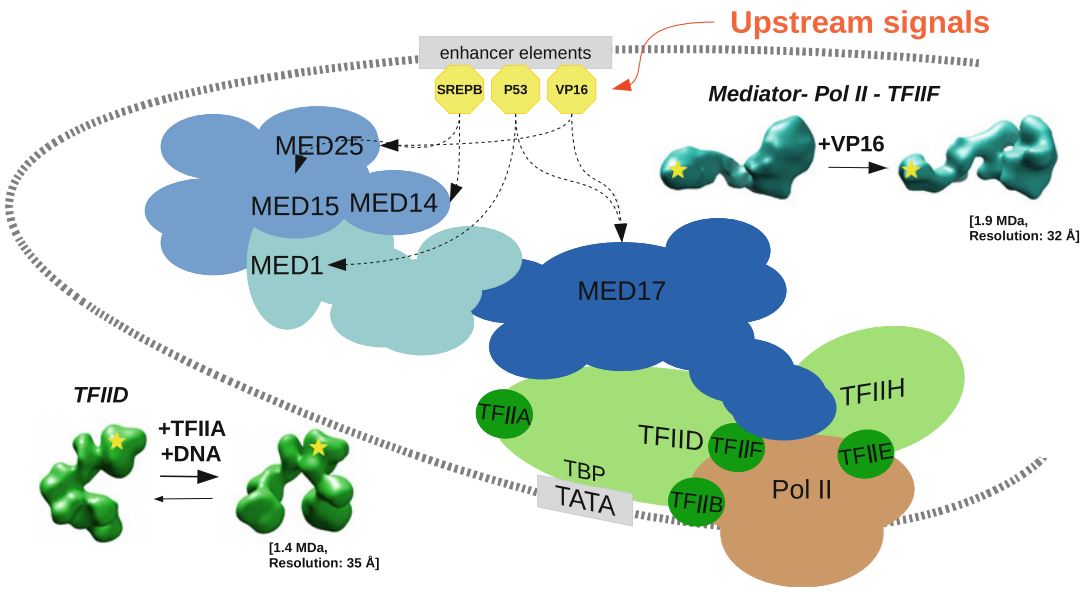

Fig. 20.5 Allosteric regulation in the transcriptional machinery. Schematic figure of the human PIC. Mediator complex (*blue particles*) has multiple binding sites for transcription factors (*e.g.*, p53, SREB and VP16). The binding of transcription factors or DNA to PIC subunits may cause structural shifts which leads to specifically regulated transcription. For example binding of VP16 transcription factor results in a structural shift in Mediator-Pol II-TFIIF assembly (*blue* cryo-EM maps; EMD-5344, EMD-5343 [52]. Cryo-EM maps (*green*) indicate directed reorganization of TFIID (EMD-2287, EMD-2284, respectively). The TFIID complex may exist in two distinct conformations, and binding of promoter DNA (TATA) and TFIIA stabilizes one of the conformations, which is competent to recruit Pol II [45]. *Yellow stars* indicate corresponding regions of the cryo-EM maps (*TBP* TATA binding protein)

[40]. This model suggests that the Mediator is best described not only as a loose network of interacting proteins but rather as a sophisticated multi-subunit complex with a network of different allosteric states. This mechanism helps to generate promoter specific outcomes through the PIC, which is comprised of ubiquitous components [41, 42]. Structurally explored examples are the sterol regulatory element binding protein (SREBP), p53 or the viral VP16 transcription factors that cause distinct structural shifts in the Mediator (Fig. 20.5). For p53, two of its domains may interact with two Mediator subunits, but interestingly only one binding mode brings about conformational changes that are compatible with Pol II elongation. The mechanism of Pol II activation is started with the binding of p53 activation domain to MED17, which in turn promotes TFIIH-dependent Pol II phosphorylation. Ultimately, the transcription machinery is now brought into its elongation competent state and transcription will start [43].

TFIID is also part of the PIC and it is composed of TATA-box-binding protein (TBP) and 13 TBP-associated factors (TAFs). Based on cryo-EM analysis the core-TFIID consists of two symmetric copies of TAF4, TAF5, TAF6, TAF9 and TAF12. In response to upstream signals, the TAF8–TAF10 complex is imported into the nucleus by importins, binds to the core-TFIID and breaks its symmetry. This results in an asymmetric 7TAF complex with new binding surfaces for six more TAF subunits and for TBP (canonical form) [44]. The promoter DNA and TFIIA trigger further structural changes and participate in the stabilization of the rearranged holo-TFIID complex (Fig. 20.5). The formation of the rearranged TFIID-TFIIA-DNA complex is then followed by binding of TFIIB, Pol II, TFIIF, TFIIE, and TFIIH to yield the transcriptionally competent pre-initiation complex [45].

For large multi-subunit complexes, single-particle electron microscopy (EM) is an essential method, especially when the sample is available in

very little amounts. This technique combined with atomic resolution structures on monomers can give pseudo-atomic models on large complexes [46].

20.7 Conclusion

There are myriads of signaling complexes in action when cells respond to their environment. Fortunately, binary protein-protein interaction data on the proteome level is rapidly increasing thanks to systematic, large-scale protein-protein interaction studies and databases [47]. To what extent this wealth of information can be harnessed for mechanistic understanding of signaling complexes greatly depends on the reconstruction of functionally important signalosomes for structural analysis.

Obtaining atomic resolution structural information about a signaling question will require the reduction of a bigger complex into biochemically well-behaving smaller units, which have less disordered regions and are conformationally less heterogeneous. In this case the pitfall could be that higher-level biochemical properties of the whole complex may be lost in a reduced system. Fortunately, these smaller complexes could be built into low-resolution maps of bigger complexes. Ultimately, signaling complexes may be visualized *in cellulo* by super resolution microscopy techniques that are capable of breaching the 250 nm light diffraction limit by an order of magnitude [48]. This potentially bridges the resolution gap between structural reconstructions by X-ray crystallography/single-particle cryo-EM/cryo-electron tomography and the visualization of fluorescently labeled protein complexes via classical light microscopy in cells.

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