Antagonistic regulation controls clathrin-mediated endocytosis: AP2 adaptor facilitation vs restraint from clathrin light chains

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Abstract

Orchestration of a complex network of protein interactions drives clathrin-mediated endocytosis (CME). A central role for the AP2 adaptor complex beyond cargo recognition and clathrin recruitment has emerged in recent years. It is now apparent that AP2 serves as a pivotal hub for protein interactions to mediate clathrin coated pit maturation, and couples lattice formation to membrane deformation. As a key driver for clathrin assembly, AP2 complements the attenuating role of clathrin light chain subunits, which enable dynamic lattice rearrangement needed for budding. This review summarises recent insights into AP2 function with respect to CME dynamics and biophysics, and its relationship to the role of clathrin light chains in clathrin assembly.

Keywords

Clathrin-mediated endocytosis; clathrin adaptors; membrane traffic; protein self-assembly; membrane biophysics

Introduction

Decades of research have yielded rich and detailed knowledge of clathrin-mediated endocytosis (CME), probably one of the best studied pathways in membrane traffic [1]. It has become apparent that CME results from orchestration of interactions between a large number of individual players. Clathrin, the central component in CME, assembles into polygonal lattices when recruited by adaptor protein (AP) complexes to cellular membranes in order to enclose cargo-laden membrane into clathrin-coated vesicles (CCVs) for intracellular transport. The AP2 adaptor complex recruits clathrin specifically to the plasma membrane, where it promotes clathrin coat assembly and uptake of associated cargo. The AP2 paralogues AP1 and AP3 act in clathrin-mediated membrane traffic within the endosomal and trans-Golgi network [2; 3], while AP4 and AP5 are involved in membrane traffic in a clathrin-independent fashion [4; 5].

Coupling clathrin recruitment and polymerisation to either cargo recognition or to membrane curvature generation are widely viewed as the key functions of clathrin adaptors. Cargo binding can be achieved by AP complexes themselves and indirectly through cargo binding partners such as β-arrestin and Dab2 amongst others. Adaptor complex influence on vesicle formation is achieved by their direct influence on clathrin assembly, as well as via binding partners such as AP180, or by recruitment of so-called endocytic accessory proteins (EAPs) such as epsin, amphiphysin and others, including those that sense and promote curvature. For an overview of EAPs participating in CME see recent reviews [1; 6]. Complete loss or mutation of individual adaptors or EAPs usually fails to abolish CME completely, but instead leads to dysfunction and/or dysregulation that often manifest in phenotypes confined to a specific tissue or organ [7-9]. This phenomenon can be attributed to the complexity of the CME machinery, as well as its ability to adapt to tissue- [10; 11] and cell type-specific needs [12; 13] through variation of participating components. The coordinated complexity and overlapping activity of CME participants makes it challenging to dissect roles of and contributions from individual components of the CME machinery. Even the contribution of clathrin itself to membrane deformation, and its mode of action in vesiculation are still subject to current debate [14]. Recent models describe CME as a coordinated collection of low affinity interactions between clathrin, adaptors and EAPs [15].

Historically, AP2 was discovered as a stimulator of clathrin assembly [16; 17] and then established as having essential cargo-recognition activity needed for cargo sequestration [18]. Recent data from state-of-the-art super resolution and cryo-electron microscopy, as well as live-cell imaging experiments further demonstrate that AP2 is a lynchpin for regulation of clathrin-coated pit (CCP) dynamics [19-24], as well as master regulator of

clathrin coat assembly at the plasma membrane [25-27]. As such, AP2 plays a key role at all stages of CME, ranging from CCP initiation [28] to maturation of a budded CCV [19]. It has become clear that the interactions with various CME components induces conformational changes and posttranslational modifications in AP2 that influence its interaction network and subsequently CME dynamics.

Furthermore, the role that AP2 plays in facilitating CME counteracts and coordinates with in-built assembly constraints conferred by clathrin light chain subunits (CLCs). The CLCs, which are obligate and tightly bound subunits of the ubiquitous clathrin found in all eukaryotes, control clathrin self-assembly through attenuation of clathrin heavy chain (CHC) interactions, simultaneously regulating lattice formation and enabling rearrangement [12]. They also serve as a link between clathrin and the actin cytoskeleton through binding actin modulators [10; 29; 30]. In vertebrates CLCs are encoded by two different genes *CLTA* and *CLTB* (in humans), and the resulting isoforms (CLCa and CLCb) have been found to play unique but cooperative roles in clathrin-mediated pathways that influence cargo signaling [31], cell migration [32], cell polarity [10] and synaptic vesicle formation [12]. Both vertebrate CLC isoforms undergo tissue specific splicing affecting biophysical properties that influence clathrin lattice formation [12]. Analysis of neuronal function in mice lacking either CLC isoform revealed that synaptic vesicle recycling requires a balance of the two isoforms and their splice variants such that animals lacking CLCa have reduced synaptic vesicles while CLCb-depleted mice produce an excess [12]. The emerging picture of vertebrate CLC isoform diversity is that their conserved differences cooperate to modulate tissue-specific clathrin functions by controlling lattice formation [12], as well as through interaction with isoform-specific partner proteins [10].

Collectively, these new insights paint a picture where AP2 and CLCs serve as important nodes for clathrin assembly regulation and enable functional plasticity of CME. This review aims to highlight recent advances in our understanding of AP2's and CLCs' contribution to CME in structural and biophysical terms, and to offer a fresh perspective on the dynamic regulation of CME.

AP2 structural changes regulate CME initiation

The AP2 complex is composed of four subunits α2, β2, μ2 and σ2 [3; 33], where the N-terminal parts of the larger (~100 kDa) α2- and β2-subunits form the core of the adaptor complex (Figure 1). Assembly of the complex requires chaperone AAGAB, without which the α2-subunit readily aggregates in absence of other subunits [34]. Unstructured hinge regions

connect the N-terminal core to the C-terminal appendage domains (also referred to as 'ear' domains) of both the α2- and β2-subunit. Nested inside the AP2 core [33] are the smaller μ2- and σ2-subunit (~50 and ~17 kDa, respectively), which are important for external regulation, cargo binding, and mediate conformational changes in the AP2 complex [35-37]. *In vitro*, the β2-subunit alone is sufficient to promote clathrin assembly [38] and comprises clathrin binding sites in the appendage region [39-42] and another in its hinge region. Complementary binding sites in the clathrin triskelion are located in the ankle region and the clathrin terminal domain (TD) (Figure 2a), and both binding sites in AP2 are necessary for efficient clathrin recruitment *in vivo* [37; 40].

AP complexes differ in their binding preference for lipids localized to the cytoplasmic face of various cellular membrane compartments. AP2 is localised to the plasma membrane through its selective binding affinity for phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), a characteristic lipid marker of the plasma membrane and endocytic sites in particular [43-45]. Indeed, dephosphorylation of $PI(4,5)P_2$ in CCVs is suggested to drive AP2 dissociation prior to uncoating [46]. Cytosolic AP2 adopts an inactive 'closed' conformation, where cargo and clathrin binding sites are buried inside the AP2 core (Figure 1). $PI(4,5)P_2$ binding triggers a conformational change in AP2 towards an intermediate open conformation, which exposes the cargo binding sites on the μ2- and σ2-subunit [47] and primes AP2 to scan the plasma membrane for relevant cargo. Cargo is recognised via distinct linear sequence motifs, such as the classic tyrosine-containing signal (YxxΦ or NPXY) or a di-leucine motif (D/ExxLL/I or DXXLL; reviewed in [48]). Disruption or introduction of AP2-recognition motifs are associated with disease states. Familial hypercholesterolemia was the first example of a disease caused by mutation in an AP2-recognition motif [49]. Recent unbiased analysis of random mutations in several disease states showed a high frequency of mutations leading to AP2 binding that results in inappropriate sequestration of transmembrane receptors into the clathrin-mediated endocytic pathway [50]. Cargo binding induces a fully open conformation of AP2, exposing its clathrin binding site on the β2-subunit hinge domain, thereby creating the causal link between cargo recognition and clathrin recruitment [37; 51] (Figure 1). Early CCP initiation factors Eps15/R and FCHO1/2 in particular recruit AP2 to the plasma membrane and assist in the conformational transition of AP2 into its open state to facilitate CCP formation [52].

Phosphorylation of AP2 and clathrin regulates CME dynamics

Recent studies show that following the conformational changes in AP2 triggered by membrane and cargo interaction that initiate CCP formation, phosphorylation of the AP2 μ2-

subunit is important for subsequent CCP maturation. Phosphorylation is facilitated by the Numb-associated kinase (NAK) AAK1 [53; 54], LRRK2, and potentially BMP2K [55] (preprint) and appears to stabilise the AP2 open state [19], as well as to influence the binding of partner proteins. Binding of NECAP proteins to phosphorylated AP2 (pAP2) is required to efficiently drive CCP maturation towards completion, possibly via subsequent recruitment of SNX9 and secondary, membrane remodelling factors [19-21]. Hence, in addition to AP2's role in cargo binding and CCP initiation, it appears that AP2 phosphorylation serves as a platform for protein-protein interactions to accelerate CCP maturation. A recent genetic screen in *Caenorhabditis elegans* found that NIMA-related kinases regulate CME dynamics that depend on AP2 activation, but possibly not via the NECAP pathway [56]. Furthermore, the 100 kDa AP2 subunits comprise other phosphorylation sites with functional roles not yet fully defined [57; 58], suggesting additional roles for phosphorylation in regulating AP2 activity.

Functional importance of AP2 μ2 phosphorylation has recently been highlighted in brain. Two independent studies identified a direct interaction between AP2 and the ROC (Ras of complex proteins) domain of LRRK2, and demonstrated that this interaction mediates AP2 μ2-subunit phosphorylation [59] (preprint) [60]. Perturbation of AP2 phosphorylation via this pathway, such as through the Parkinson's Disease (PD)-associated mutation that upregulates the activity of LRRK2, leads to dysfunctional CME in the brain. Interestingly, the EAPs synaptojanin and auxilin are also phosphorylation targets of LRRK2 and their mutations are PD-associated risk factors [61-63]. Further evidence for the role of AP2 phosphorylation in PD and CME is suggested by a recent study [64] that reports a role for pAP2 association with the PD risk factor α-synuclein and $PI(4,5)P_2$ during CME of synaptic vesicle proteins. Together, these newly identified AP2 interaction partners in PD highlight a role for AP2 phosphorylation as a key protein-protein interaction modifier and underscore the importance of CME for brain function. Interestingly, NECAP isoforms, α-synuclein, LRRK2 and NIMA-related kinases all exhibit tissue-specific expression, suggesting tissue-specific roles for these particular proteins in controlling CME. This variation of pAP2 interaction networks apparently modulates the CME pathway for specialised functions in differentiated tissue.

Phosphorylation of clathrin subunits in response to receptor signalling has also been implicated in regulation of CME dynamics. While the mechanism remains unknown, CHC phosphorylation by tyrosine kinases correlates with balancing cargo localisation between conventional CCPs and clathrin coats associated with membrane raft domains [65; 66].

Recently, CLCs have been identified as a phosphorylation target for CME of a subset of G protein-coupled receptors (GPCRs) which depend on G protein-coupled receptor kinase 2 (GRK2) for internalisation [67]. Maib and colleagues observed that cells expressing a mutant CLCb lacking all potential phosphorylation sites are impaired in CCP progression and slow in exchange of clathrin at CCPs [68]. This suggests a novel regulatory mechanism for controlling GPCR uptake that depends on how CLC phosphorylation affects CCP maturation, in addition to the well-characterised recruitment of β-arrestin, a CHC- and AP2-interacting EAP, that is triggered by GRK phosphorylation of GPCR upon ligand binding [69].

The controversial biophysics of CME

Transient interactions of AP2 with a range of accessory proteins are a hallmark of the progression of CCP maturation [52]. Hence, the sum of many specific, low-affinity interactions and their orchestration within a complex protein interaction network regulate CME and allow diversification, specialisation and adaptation of CME in different tissues [15]. A number of approaches have been taken to define the biophysical contributions of individual components in facilitating clathrin assembly and membrane deformation (i.e. CCP maturation). Experimental, computational and theoretical studies have estimated the energetic costs of membrane deformation, and how they may be afforded by clathrin polymerisation and adaptor recruitment to CCPs [70-73]. However, the underlying energetic and biological assumptions of these models are still being defined by the field. Consequently, addressing the question of whether the two major coat components themselves, namely AP2 and clathrin, play a passive or active role in membrane deformation during the process of CCV formation has generated several models.

Probably the most infamous of these controversies is the question of whether clathrin assembles into polygonal lattices statically and irreversibly at constant curvature, or whether clathrin assembly is a more dynamic and flexible process that allows for lattice rearrangement and gradual invagination of a growing CCP (see for example a recent review by Sochacki and colleagues [14]). While both scenarios lead to the same outcome, the underlying biophysical conditions are significantly different – such as strong vs weak intermolecular binding events, for example. Thanks to recent advances in characterising the relative spatiotemporal recruitment and enrichment of CME components throughout the life span of a CCP [22], as well as in visualising *in vivo* morphology [25; 74], membrane deformation can be considered as a continuum, and the biophysical contributions of clathrin and AP2 to CCP maturation can be evaluated in molecular detail [14]. In addition, *in vitro* studies of clathrin assembly [12] and recent cryo-electron microscopy studies [26; 27; 75; 76] have contributed new perspectives on the mechanics of coat assembly. Overall,

evidence is accumulating that curvature transition occurs during CCP maturation at least to some extent in cells [22; 24; 25; 74]. In light of these advances and new perspectives on clathrin light chain (CLC) functions [12], the relative contributions of clathrin subunits and AP2 to the mechanics and energetics of assembly and maturation have become more evident, as discussed in the next section. Whether lattice rearrangement is a net energy consuming process and facilitated by the uncoating ATPase Hsc70 and its co-factors such as GAK and auxilin, as recently suggested [68; 77], is still unclear [78].

Antagonistic mechanics mediate clathrin-coated vesicle formation

The CLC subunits are bound to the central (hub) domain of the clathrin triskelion (Figure 2a) formed from three clathrin heavy chains (CHCs; in humans, CHC17). The alpha-helical CHC-binding domain of CLCs is flanked by an N-terminal region that comprises conserved binding sites for actin-modulating proteins [10; 79], and a C-terminal region that reinforces the stability of the CHC trimer [12; 80]. The presence of CLCs inhibits spontaneous clathrin assembly at physiological pH [81; 82], thereby putting assembly under the control of adaptors that recruit clathrin to cargo-laden membrane and linking clathrin coat assembly to cargo sequestration. CLCs' inhibition of clathrin assembly is achieved by their influence on the conformation of the triskelion knee region through binding of the flexible CLC N-terminal domain [12; 83; 84]. When the triskelion knee is engaged by the N-terminal region of CLCs, the resulting CHC knee angle is unfavourable for lattice formation, and during assembly this CLC region retracts [83], rendering the knee more flexible. The assembly-attenuating interaction between the CLC N-terminus and the CHC knee is transient and reversible, while the association between the CHC proximal leg and the CLCs' central CHC-binding domain is high-affinity and stable [85; 86]. Thus, CLCs put a brake on how readily triskelia "fit" into growing clathrin lattices. In energetic terms, CLCs can be considered to lower the binding interaction between triskelion legs, and conversely raise the energy barrier (i.e. critical clathrin concentration) for spontaneous assembly to occur [87] (Figure 2b). Neuronal splice inserts near the CLC C-terminus further influence CHC lattice properties. In a recent study, clathrin comprising a single neuronal CLC splice variant was less able than clathrin with the corresponding non-neuronal CLCs to deform mebrane *in vitro*. However, clathrin comprising neuronal CLCa and neuronal CLCb was found to work in synergy to facilitiate membrane deformation *in vitro* and was required for normal synaptic vesicle recycling *in vivo* [12]. Within the clathrin lattice, the 18-30 amino acid CLC neuronal inserts (adjacent to the CHC trimerization domain) are predicted to be in the vicinity of the CHC knee of a neighbouring triskelion. Thus, by interacting directly with a neighbouring CHC knee or with the N-terminus of a CLC associated with the neighbouring CHC, the neuronal splice inserts could further influence knee flexibility [12] to affect assembly behaviour and lattice properties. Indeed,

CLC diversity had no modulatory effects on lattice assembly when the triskelion was severed at the knee into its central hub and distal fragments to produce ultimate knee flexibility [12].

The conformational limitations imposed by CLCs on clathrin triskelia are overcome by antagonistic influences of adaptors such as AP2 that promote assembly *in vitro* (Figure 2b). It has been previously proposed that this is achieved by adaptor complexes being able to simultaneously bind multiple clathrin legs, which could facilitate the alignment of clathrin legs for assembly [16; 42; 88]. Clathrin can bind to the β2-subunit of AP2 in both the hinge region and the appendage domain (Figure 2a). Two binding sites have been mapped in the β2-appendage domain, which interact with the clathrin ankle domain [40; 41], and a so-called clathrin box motif in the hinge region is recognised by the CHC terminal domain (TD) [89; 90]. The combination of multiple CHC interactions with the appendage and the hinge domains would, through reciprocal crosslinking, contribute considerable stability to the region of the lattice where these interactions take place (Figure 2a). Indeed, the presence of AP2 confers stiffness to clathrin cages, as measured by atomic force microscopy [91; 92].

Studies of purified clathrin assemblies with the β2-hinge-appendage domain showed that both the hinge region and appendage domain are needed to promote efficient *in vitro* clathrin assembly in the absence of membrane [33], consistent with a role for AP2-driven crosslinking in clathrin polymerisation (Figure 2a). This concept was supported by the requirement for a CHC fragment comprising both the TD and ankle domains to reconstitute clathrin cage assembly of recombinant CHC fragments driven by the β2-hinge-appendage [88]. From these studies, it was proposed that crosslinking results in formation of a 'counter-hub' that is a trimer of the distal CHC leg segments, aligning them with the proximal leg segments, in order to promote clathrin assembly. This counter-hub alignment at the β2-appendages is visible in cryo-electron tomography (cryo-ET) structures of both reconstituted and native clathrin-coated membranes (Figure 2c). These two recent cryo-ET studies confirm the β2-appendage makes contacts with different sites in the ankles of two separate clathrin legs, and reveal additional contact between a third clathrin leg by the β2-appendage at an unpredicted site in the CHC TD. The unstructured nature of the hinge regions [33] impairs their visualisation by averaging methods used for cryo-ET analysis.

Notably, the β2-appendages are not uniformly distributed throughout these assembled clathrin structures, but appear more frequently at hexagonal vertices (Figure 2c) rather than vertices of curvature-generating pentagonal faces [26; 27]. *In vitro*, clathrin assembles spontaneously into membrane-free polygonal 'cages' of sizes similar to CCVs observed in

cells comprising twelve pentagons for closure [12; 84]. In cells, clathrin favours assembly at membranes of high curvature [93], and upon release of membrane tension, flat clathrin assemblies gain curvature without apparent external energy input [74; 94]. This suggests that clathrin lattices favour curvature, and that flat clathrin lattices are energetically less favourable than curved lattices (Figure 2d). However, lattice rearrangement comes at an energetic cost, as bonds formed between clathrin legs need to be broken to allow rearrangement. Thus, flat lattices, although energetically less favourable than curved lattices, may initially form on membrane and be stable as they may be 'trapped' in a local energy minimum (Figure 2d). The energy barrier to reach the global minimum and thermodynamically stable form of curved lattices is lowered by CLCs, as CLCs increase triskelion solubility by stabilising CHC knee conformations unfavourable for lattice incorporation. Thus, CLCs function during both nucleation and maturation of CCVs by limiting CHC-CHC interaction; they impede spontaneous assembly during nucleation, and support lattice rearrangement during maturation by lowering the energy barrier ΔE_r for the necessary lattice rearrangement. The latter role is further supported by the observation that assembly of CHC lacking CLCs is poor at membrane deformation in a liposome model of clathrin budding that requires lattice rearrangement [12; 84] and in line with a recently proposed model where CHC legs are less well aligned in flat lattices than in curved lattices [74]. It should also be noted that the presence of CLCs generally maintains rigidity and orientation of the CHC legs [84; 95], reducing available assembly interaction faces.

Nucleation of clathrin assembly at membranes by reciprocal crosslinking with AP2 [28] could energetically assist assembly into hexagons and counteract CLC constraint by promoting counter-hub formation underneath knee regions, possibly shifting the CLCs away from their knee interaction (Figure 2c). The multi-site crosslinking that the combination of hinge and appendage binding engenders (Figure 2a) would also energetically enable formation of a hexagonal clathrin lattice at membranes, and might counteract clathrin's tendency to curvature during nucleation (Figure 2d). As the coat matures and a threshold of recruitment and productive nucleation is reached, the introduction of curvature (pentagons) would be favoured by the intrinsic curvature of clathrin lattices, but sterically less accommodating of AP2-appendage binding, as indicated by comparing recent structures of the two vertices (Figure 2c). Subsequent replacement of CHC-appendage interactions with EAPs due to pentagon formation would represent a checkpoint for CCP maturation, enabling curvature and membrane deformation. At this stage, the ability of CLCs to attenuate lattice assembly also lowers affinities of CHC leg interactions further favouring lattice rearrangement (Figure 2d). This scenario for nucleation and rearrangement depends on low-affinity individual interactions to maintain flexibility for lattice rearrangement and to decrease the probability of

permanently incorporating lattice defects [96]. Thus, the balanced energetics of CLC- and AP2-regulated clathrin-clathrin interactions could drive vesicle formation (Figure 2d).

Recent data shows that the hinge domain alone is able to promote clathrin assembly at artificial cargo-loaded membranes without the β2-appendage domain [26], in contrast to the β2-appendage domain's necessary involvement in cage assembly without membranes [39]. However, the behaviour in the reconstituted system might be explained by hinge-mediated recruitment raising the local surface concentration of clathrin and does not preclude an active role for the appendage domain in cellular CCP initiation, where the assembly conditions are different. In this context, it is notable that the β2-appendage-clathrin interaction alone appears to be insufficient for clathrin recruitment [37]. An *in silico* model of AP2's role in CCP maturation suggests that efficient growth of CCPs depends on maintaining proximity of the AP2 core membrane binding site and AP2's clathrin binding site [97], which the flexible hinge region may help to accommodate.

Together, the studies described in this section suggest a model (Figure 3) in which AP2-appendage-CHC binding along with β2-hinge-TD binding may be significantly active at the CCP nucleation stage to initiate assembly into hexagons by crosslinking and antagonise CLC inhibition. Then, the flexibility of the β2-hinge region and its role in clathrin recruitment would enable further clathrin assembly to introduce pentagons, which would drive lattice curvature and promote dissociation of the appendage domain from clathrin engaged in pentagonal faces. The free appendage domain could then engage EAPs to assist in membrane deformation, as well as promote deformation by diminishing stiffness due to AP2 crosslinking. Like the β2-hinge-TD interaction, CLC attenuation of clathrin assembly operates throughout CCP assembly and maturation, first as an antagonist requiring adaptor-mediated nucleation and then to facilitate rearrangement of the lattice to introduce curvature by reducing triskelion interaction (Figure 3). Consistent with this model, live cell imaging reveals a threshold of AP2 enrichment within a growing CCP, which precedes the maximum of clathrin accumulation and full curvature generation [25]. Furthermore, correlative light-electron microscopy (CLEM) studies show AP2 is more likely localised to the rim of a an early but not late CCP, while many EAPs partition towards the edge of a CCP [23]. In fact, flat lattices disappear upon knock down and knock out of AP2 [98] and become more prominent upon cell differention of stem cells into fibroblasts expressing AP2 at higher levels [99]. The observations that CLCs are important for efficient membrane deformation *in vitro* and *in vivo* [12; 84] are also consistent with their roles at both stages.

Conclusion

It has become clear that AP2 interaction with clathrin and beyond are important for effective CCP maturation, CME progression and CCV architecture, as well as the canonical functions of cargo recognition, coat localisation and assembly stimulation. We can now draw from a plethora of mechanistic and structural details to appreciate how this master regulation by AP2 is achieved. However, extrapolation of these findings to other adaptors, even as closely related as AP1 and AP3, remains to be established. This is exemplified by a recent study which showed that cellular overexpression of the CHC TD served as a dominant-negative factor to inhibit specifically AP2-clathrin, but not necessarily AP1-clathrin interactions [100]. Paralogues AP4 and AP5 function independently of clathrin, but in concert with Ras- and Rab-related GTPases to mediate membrane traffic at the trans-Golgi network. Given the evolutionary relationship with the clathrin-associated adaptors, it will be interesting to establish whether AP4 and AP5 play similar roles in orchestrating membrane bending in these contexts. It is thus clear that despite our breadth of knowledge of CME at the plasma membrane, how clathrin machineries function in other cellular locations and pathways is less understood. In vertebrates, there are four major isoforms of CLCs with different expression patterns in different tissues [101]. These isoforms affect lattice quality and budding efficiency differently [12], and possibly also differently at different sites in the cell. In particular, the phenotypes of CLC-knock-out mice reveal a key and distinct role for CLC isoforms in synaptic vesicle recycling from endocytic compartments, in line with other studies that demonstrate a prominent role for CLCs in cargo trafficking from endosomal compartments [102; 103]. Hence, CLCs also present variable organelle- and tissue-specific challenges to adaptor function in clathrin coat formation. Intriguingly, the role and importance of (i.e. dependency on) AP2 and CLCs in CME considerably vary with cargo (i.e. on a molecular level [68; 98]), cell type (i.e. on a physiological level [99; 101]) and organism (i.e. on an evolutionary level) ([51; 104]), underscoring the vast breadth of CME plasticity. For instance, comparing yeast and mammalian CME reveals that AP2 has evolved to become more central for vertebrate CME compared to yeast endocytosis, while CLCs are less dispensible in yeast and appear to serve a more nuanced modification of clathrin function in higher organisms. There is also a minority of cargoes that rely on different adaptors than AP2 for CME. Challenges for the future will be to dissect the structural and mechanistic differences between adaptors that link clathrin machineries to this subset of endocytic cargo and to different cellular organelles, as well as to understand how tissue-specific differences in clathrin subunit isoforms alter adaptor requirements. Redirecting focus from the plasma membrane towards the inside the cell, where membrane remodelling occurs from organelles with different morphologies, as well as characterising tissue-specific diversity of CCV

components, will be necessary to fully integrate biophysical principles with the cell biology and physiology (disease) associated with clathrin-mediated membrane traffic.

Figure 1: Regulation of the AP2 interaction network through AP2 conformational changes and phosphorylation.

The progression of AP2 conformational changes and the sequence of cumulative binding partner interactions enabled by these changes is shown (grey arrows). Cytosolic AP2 adopts a closed, inactive conformation with key lipid and protein binding sites inactive and is recruited to the plasma membrane by early initiation factors FCHO1/2 and Eps15/R (left). At this stage, the hinge-clathrin binding sites are obscured [37], and the appendage-clathrin or appendage-endocytic accessory proteins (EAP) sites are not sufficiently high affinity to contribute to clathrin recruitment or assembly nucleation [37]. Phospholipid binding allows for efficient cargo binding, which together trigger the 'open' conformation of AP2 that is able to recruit clathrin (middle). Phosphorylation of the μ2-subunit by AAK1 (dependent on open conformation), LRRK2 and potentially BMP2K, creates new binding sites that serve as an interaction hub for various other EAPs that facilitate CCP maturation (right). CCV uncoating and phospholipid dephosphorylation promotes dissociation of AP2 from the membrane to restore the cycle. The phosphatase involved in AP2-dephosphorylation is currently unknown. The α2- and β2-subunits are divided into appendage and core domains linked by flexible hinge regions, as labelled. The appendage has at least two CHC-binding sites (see text), represented by a single star here, both of which can compete with EAP binding. References are provided in the text. Asterisks indicate reference to prepublication data.

Figure 2: Models of how AP2 and clathrin light chains (CLCs) regulate CME initiation and progression.

a Model of how AP2-CHC interaction facilitates clathrin crosslinking. One clathrin heavy chain (CHC, yellow) can be subdivided into terminal domain (TD, 1), ankle (2), distal leg (3), knee (4), proximal leg (5) and trimerization domain (6). Regions 4-6 are collectively referred to as the 'hub'. AP2 binding sites are located in the ankle and TD region (red stars, 7). The clathrin light chain (CLC) subunits (only CHC-binging domain depicted, orange, 8) bind along the CHC proximal leg (5) and regulate CHC knee (4) conformation, thereby attenuating clathrin assembly and regulate membrane bending efficiency *in vitro*. The appendage of the AP2 β2-subunit binds sites in the CHC ankle and possibly the TD. The hinge region binds the TD via a classical clathrin box motif. The colour coding of the AP2 subunits is the same as in Figure 1. The combination of two separate binding domains in both CHC and AP2 enables crosslinking of at least two triskelia by AP2 and conversely one triskelion can attract more than one AP2 adaptor. These mutual interactions promote clathrin assembly by alignment of the CHC legs (left) and increase the presence of AP2 at nucleation sites (right), leading to clathrin-coated pit (CCP) nucleation and clathrin polymerisation.

b Model of how CLCs and AP2 affect energy barriers to clathrin assembly. CLCs create an energy barrier that inhibits spontaneous clathrin assembly by interacting with the triskelion knee and restricting its conformation, increasing the energy barrier for assembly ΔE_a, (blue), compared to spontaneous assembly of clathrin without CLCs (grey). This is counteracted by AP2, which induces clathrin conformation(s) favourable for assembly by crosslinking, reducing the energy barrier (green). References are provided in the text.

c Clathrin conformation and AP2 β2-appendage presence at hexagonal and pentagonal faces of the clathrin lattice. Top: AP2 β2-appendage 'crosslinks' three triskelia (numbered 1,2,3) underneath an edge that is 2-3 vertices away from their centres, irrespective of the geometry of the polygonal face (triskelia crosslinked by hexagon-associated AP2 (blue) highlighted in pink and crosslinked by pentagon-associated AP2 (red) highlighted in green). Only ankle and TD domains engaged with the highlighted AP2 β2-appendages are shown for simplicity. Bottom: Cryo-EM structures of AP2-CHC interaction at pentagonal and hexagonal faces as schematically outlined above (same colour key as in top scheme, produced using UCSF-chimera from EMDB-10753 and EMDB-10752). The knee regions (K) in the grey triskelia are pointed out by arrows. Overlay of the two structures (below) reveals significant conformational differences in triskelion 3, which is closer to the TD of triskelion 1 (black arrows), introducing potential steric constraint on the presence of the β2-appendage

in pentagonal faces (highlighted by asterisk in left and middle structure), explaining a reduction in β2-appendage domains associated with pentagonal vertices.
d Model of how the energetics of clathrin-mediated CCP formation is influenced by CLCs and AP2. As in **b**, CLCs increase the initial assembly energy barrier compared to CHC-only-clathrin, while AP2 promotes assembly (reduces the energy barrier ΔE_a) during initiation of assembly (nucleation). Initial assemblies on the membrane are flat and hexagonal (low curvature). Flat lattices face a rearrangement barrier ΔE_r, which is highest in absence of CLCs because CLCs reduce triskelion interaction (supported by data showing that assembly of CHC lacking CLCs is poor at membrane deformation). Thus, flat lattices, although energetically unfavourable, are stable when 'trapped' in an energy valley. If the initial assembly barrier is low (as in presence of AP2, green), this valley may be less pronounced, thus favouring a state where fully 'productive' CCPs are more easily formed by introduction of pentagons. CLCs enable curvature generation by lowering the energy barrier ΔE_r for the necessary lattice rearrangement. During rearrangement, the β2-appendage would tend to dissociate from pentagonal vertices due to steric constraints, reducing the crosslinking that favours hexagons and promoting clathrin assembly into curved structures, contributing to membrane deformation (also enabled by recruitment of endocytic accessory proteins). References are provided in the text.

Figure 3: Antagonistic mechanics of AP2 and CLCs in the lifespan of a clathrin-coated pit (CCP).

In the initial stages of CCP formation, AP2 engages clathrin using both the β2-appendage and β2-hinge binding sites. This promotes hexagon formation (pink), aligning and crosslinking clathrin legs to overcome clathrin light chain (CLC) attenuation of clathrin assembly. At this stage, the inhibitory effects of CLCs confer dependence on AP2, which ensures clathrin assembly on membranes where cargo can be bound. When nucleation reaches a threshold of hexagonal lattice formation, the resulting recruitment of clathrin will begin to favour pentagon formation (green), which is structurally less accommodating for β2-appendage binding, allowing its release. At this stage, AP2-clathrin interactions are reduced and the β2-appendage can recruit endocytic accessory proteins (EAPs) to contribute to membrane deformation, while the β2-hinge is still active in clathrin recruitment to continue assembly. This 'relaxation' of crosslinking frees up clathrin to rearrange, a process that is facilitated by the CLCs continuing to attenuate triskelion leg interactions. The dotted lines indicate degree of curvature in the forming CCP (side). The three different triskelia forming the counter-hub underneath each vertex are distinguished by gradation of colour (surface and interior) and their associated CLCs shown in light blue (interior). The β2-hinge-appendage is shown emerging from a membrane associated AP2 core (grey, interior).

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Dedication

This paper is dedicated to the memory of Linton Traub and Ernst Ungewickell, both of whom contributed important concepts and data cited here.

Competing Interest

The authors declare no competing interest.

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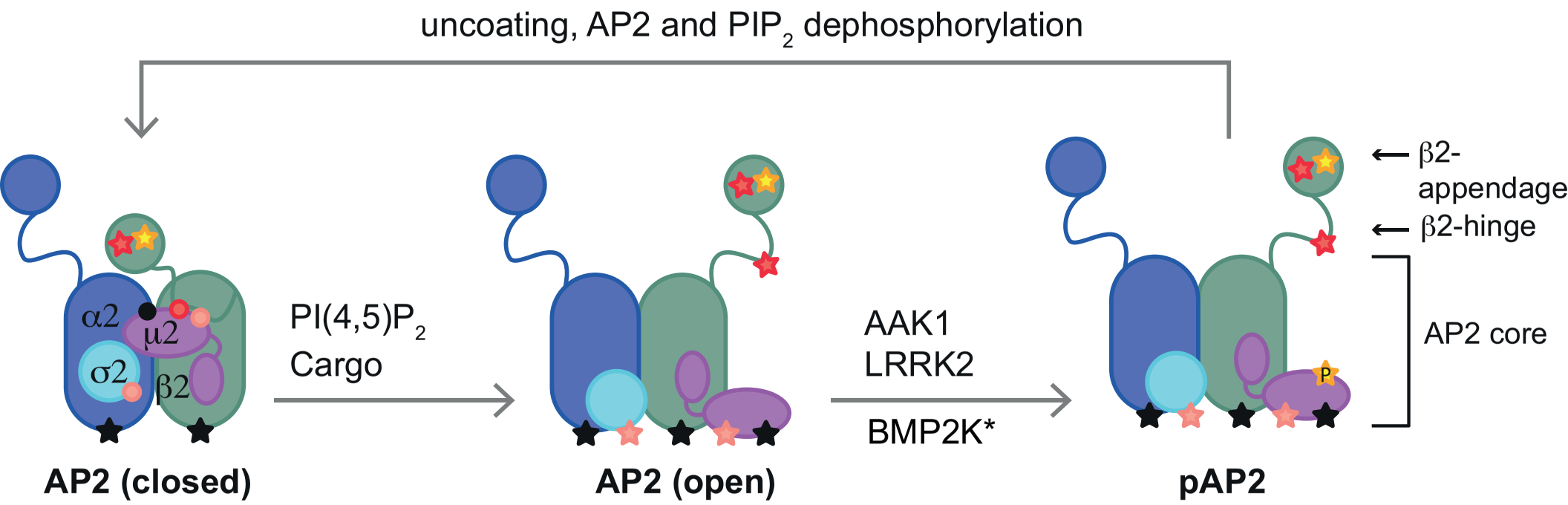


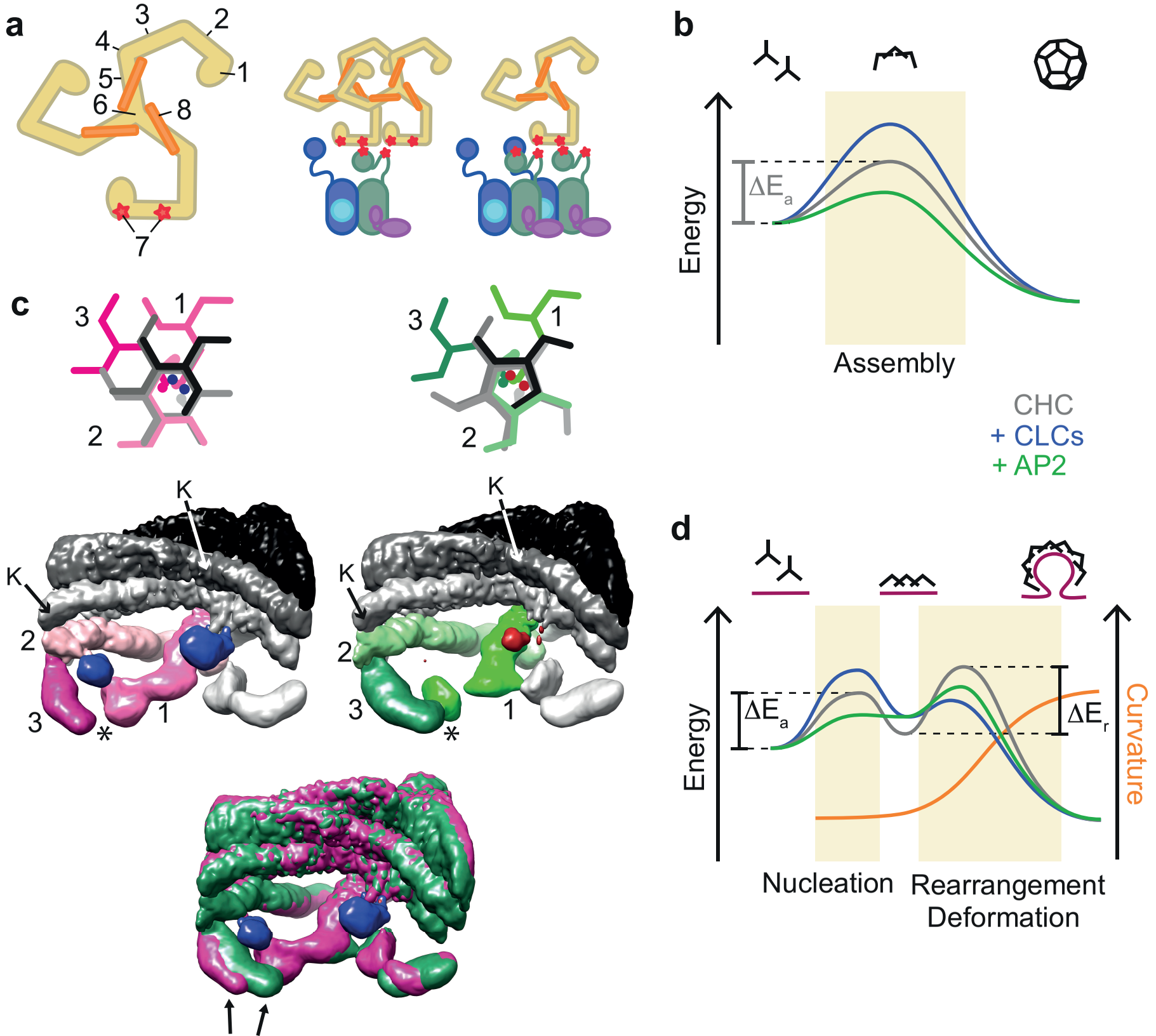
Binding partner progression

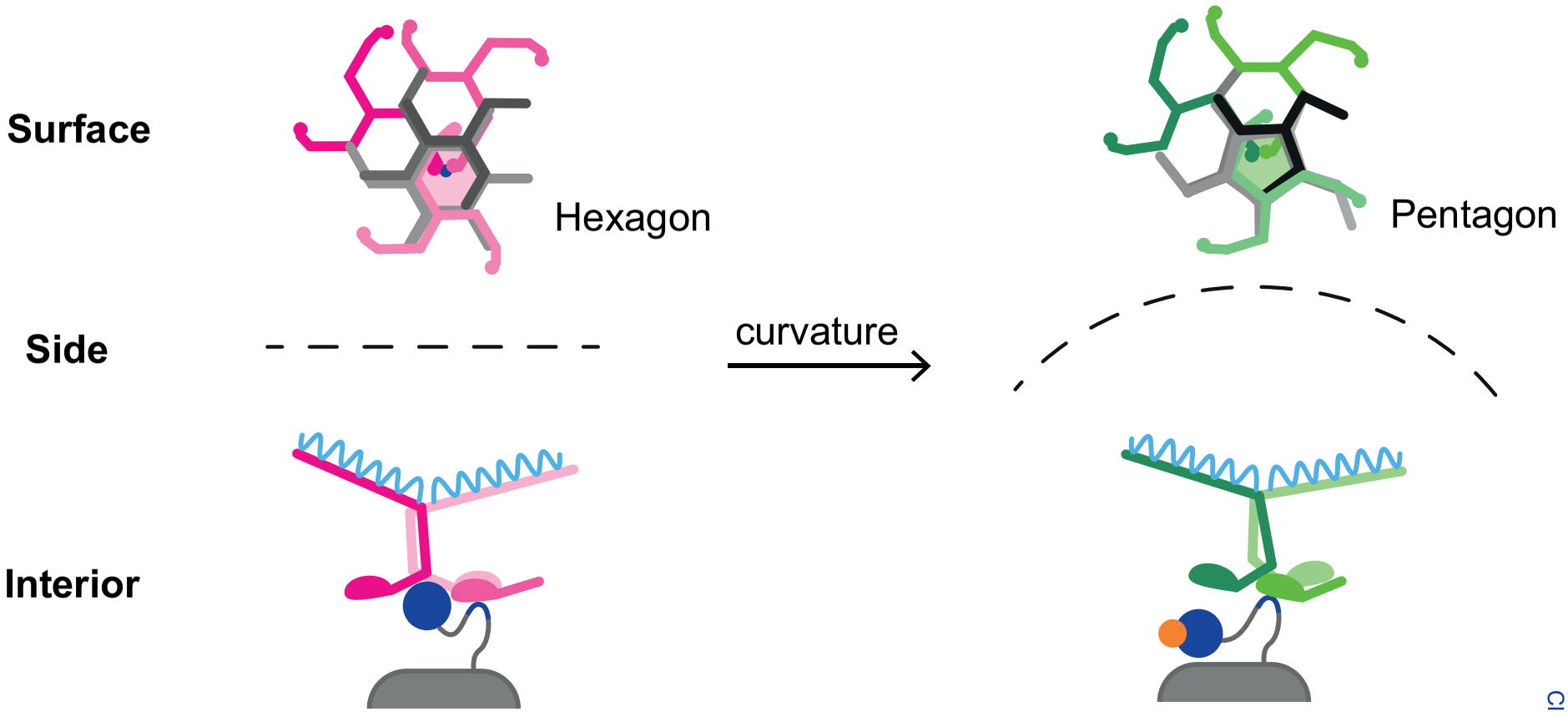
FCHO1/2
Eps15/R
→
clathrin
EAPs
→
NECAP
α-synuclein*

Binding sites

● ★ PI(4,5)P2 ● ★ cargo ● ★ clathrin ★ P EAPs

○ inactive/inaccessible ☆ active/accessible





Coat component	Role: Nucleation at the membrane	CCP maturation
β2-appendage	Crosslinking/leg alignment	EAP (●) recruitment
β2-hinge	Crosslinking/leg alignment	Clathrin recruitment
CLCs	Inhibition of random assembly	Lattice rearrangement

Credit Author Statement

Manuscript and figures were drafted by LR, based on discussions with FMB. FMB and LR jointly wrote and edited the final version.