Molecular mechanism and physiological functions of clathrin-mediated endocytosis

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Abstract | Clathrin-mediated endocytosis is the endocytic portal into cells through which cargo is packaged into vesicles with the aid of a clathrin coat. It is fundamental to neurotransmission, signal transduction and the regulation of many plasma membrane activities and is thus essential to higher eukaryotic life. Morphological stages of vesicle formation are mirrored by progression through various protein modules (complexes). The process involves the formation of a putative FCH domain only (FCHO) initiation complex, which matures through adaptor protein 2 (AP2)-dependent cargo selection, and subsequent coat building, dynamin-mediated scission and finally auxilin- and heat shock cognate 70 (HSC70)-dependent uncoating. Some modules can be used in other pathways, and additions or substitutions confer cell specificity and adaptability.

Adaptor proteins
Proteins linking receptors to clathrin triskelia.

Triskelia
Shapes that consists of three bent limbs radiating from a centre.

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Clathrin-mediated endocytosis is the uptake of material into the cell from the surface using clathrin-coated vesicles (FIG. 1). Although clathrin-coated vesicles can also be formed from other membranous compartments, the term clathrin-mediated endocytosis is used to refer only to intake through vesicles formed from the plasma membrane (BOX 1). The pathway is versatile, as many different cargoes can be packaged using a range of accessory adaptor proteins. Clathrin-mediated endocytosis is used by all known eukaryotic cells. Other clathrin-independent entry portals (such as caveolin-dependent pathways) have been described, although their molecular details and cargo specificity are not as well defined (reviewed in REF. 1) (FIG. 1).

The uptake of material inside the cell was first visualized following the introduction of glutaraldehyde fixation in the 1960s; this generated electron microscopy images of vesicles with proteinaceous coats in many different tissues[2,3]. Clathrin was then identified as being the major protein making the lattice-like coat around vesicles, which were described as "vesicles in a basket" (REF. 4).

Clathrin-coated vesicle formation proceeds through five stages that correspond to ultrastructural and cell biological observations: initiation, cargo selection, coat assembly, scission and uncoating. Following cargo selection and initiation of pit formation, soluble clathrin triskelia polymerize into hexagons and pentagons, the relative ratio of which accommodates a wide range of membrane curvatures; synaptic clathrin-coated vesicles require approximately 100 triskelia. Clathrin does not bind directly to the membrane or to cargo receptors and thus relies on adaptor proteins and complexes (such as adaptor protein 2 (AP2)) and accessory proteins (such as AP180 and epsin) to be recruited to the plasma membrane (TABLE 1). As with clathrin, all of the additional accessory proteins are cytoplasmic proteins that are recruited to sites of vesicle budding and, after the vesicle is formed, are recycled back to the cytoplasm for reuse in another cycle of endocytosis (FIG. 2a). Although the endocytosis of cargo receptors can be stimulated by ligand binding (for example, epidermal growth factor receptor (EGFR)), other receptors (for example, transferrin receptor (TfR)) are constitutively internalized[5]. When cargo has been taken up, it is sorted in endosomes and either sent back to the surface or targeted to more mature endosomes and later compartments (such as lysosomes and multivesicular bodies)[6] (FIG. 1).

In keeping with cargo versatility, clathrin-mediated endocytosis has a range of different functions. These include: regulating the surface expression of proteins; sampling the cell's environment for growth and guidance cues; bringing nutrients into cells; controlling the activation of signalling pathways; retrieving proteins deposited after vesicle fusion; and turning over membrane components by sending these components for degradation in lysosomes.

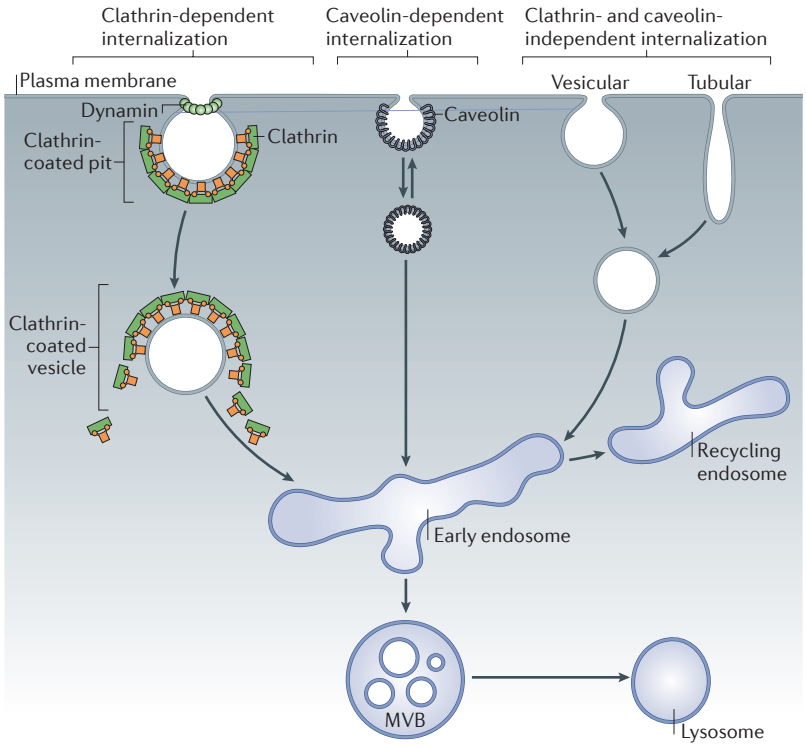


Figure 1 | **Clathrin-dependent and -independent internalization pathways.** There are multiple pathways of endocytosis into cells; for example, clathrin-dependent, caveolin-dependent and clathrin- and caveolin-independent internalization. Internalized cargo is trafficked into endosomes, where it is sorted either back to the surface of the cell or into other compartments (multivesicular bodies (MVBs) and lysosomes) for degradation. Clathrin-mediated endocytosis of cargo destined for endosomes is well characterized. Caveoli work in parallel, but their specific cargoes are not well defined. Further molecular characterization is required to define the clathrin- and caveolin-independent pathways, some of which are vesicular and others are tubular.

In this Review, we highlight the fundamental nature of this pathway, which exists to select and gather together many different proteins into the one vesicle and to regulate cellular responses. We demonstrate the modular nature of the pathway, which allows it to have distinct functions in different parts of the cell and in different cells. Finally, we stress the fundamental role of clathrin-mediated endocytosis in cellular functions and discuss the low representation of mutations and deletion of its components in human diseases.

The clathrin-coated vesicle cycle

Ultrastructural and cell biological observations have defined the five stages of clathrin-coated vesicle formation (FIG. 2).

Nucleation. Morphologically, the first stage of vesicle budding involves the formation of a membrane invagination called a pit. Clathrin-coated pit initiation was traditionally thought to be triggered by the recruitment of the highly conserved protein AP2 to the plasma membrane. This can be mediated through its binding to endocytic motifs present in cytoplasmic tails of receptors[7] and to the plasma membrane-specific lipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2)[8]. However, recent studies in yeast and mammalian cells indicate that the initiation stage may involve the formation of a putative nucleation module that defines the sites on the plasma membrane where clathrin will be recruited and vesicles will bud[9,10]. This putative nucleation module is thought to assemble only at the plasma membrane because of a preference for PtdIns(4,5)P_2. It includes FCH domain only (FCHO) proteins, EGFR pathway substrate 15 (EPS15) and intersectins[9–11] (FIG. 2a,b), and is thought to be required for clathrin-coated pit formation, as depletion of either FCHO proteins or EPS15 and intersectins has been shown to inhibit clathrin coat recruitment[9–11] (FIG. 2c). The F-BAR domain of FCHO proteins can bind to very low curvatures, and its membrane-bending activity is required for progression of the clathrin-coated pits[10,11], suggesting a need for membrane curvature generation even before clathrin recruitment (BOX 2).

Cargo selection. The proteins of the putative nucleation module are then thought to recruit AP2 (REF. 10), which, together with other cargo-specific adaptor proteins (TABLE 1), mediates cargo selection. After clathrin, AP2 is the most abundant component of clathrin-coated vesicles[12]. It specifically acts at the plasma membrane, but similar protein complexes (AP1, AP3 and potentially AP4) are found associated with clathrin-coated vesicle formation on intracellular membranes[13]. AP2 binds both cargo and PtdIns(4,5)P_2; it interacts directly with motifs in the cytoplasmic tails of transmembrane receptors through its μ-subunit and σ-subunit, and indirectly with cargo using its appendage domains to bind accessory adaptor proteins[14,15].

A wide range of plasma membrane accessory adaptor proteins has been identified in different cell types that bind to different receptors[16]; for example, stonin 2 recruits synaptotagmin[17], Dishevelled binds Frizzled[18], and HRB recruits vesicle-associated membrane protein 7 (VAMP7)[19]. These cargo-specific adaptor proteins always bind the core adaptor AP2 (REFS 20,21) (FIG. 2b). FCHO proteins also have a putative ligand-binding domain[10,11], and thus cargo selection might start as early as the nucleation stage. Furthermore, the AP180 amino-terminal homology (ANTH) and epsin N-terminal homology (ENTH) domains are membrane-binding and membrane-bending modules, respectively[22,23] (BOX 2), but they are also thought to be involved in cargo binding[24,25]. As an extension, we predict that all membrane-interacting molecules in clathrin-mediated endocytosis are both cargo adaptors and curvature effectors (BOX 2). This would ensure curvature generation regardless of which cargo is selected.

Because AP2 binds clathrin and most of the accessory proteins, it acts as a major hub of interactions in the maturing clathrin-coated pit (FIG. 2a) and is the last stage that is observed exclusively during clathrin-coated vesicle formation from the plasma membrane, as downstream components, such as clathrin, dynamin and uncoating proteins, are also found in clathrin-coated vesicle budding events from other membranes. If maturation of the complex by recruitment of AP2 is prevented, then the nucleation complex is still assembled but clathrin is not recruited and vesicles do not form[10,26,27] (FIG. 2c), which indicates a

Module
A set of proteins working together to carry out a specific function.

F-BAR domain
FES–CIP4 homology (FCH) Bin–amphiphysin–Rvs (BAR) domain.

Box 1 | **Setting limits on clathrin-mediated endocytosis**

There is some recurring confusion about the nature and extent of clathrin-mediated endocytosis in metazoans. The following points are important in this context.

Clathrin function is not limited to endocytosis
Clathrin is crucial in many other cellular processes, such as endosomal sorting complex required for transport (ESCRT)-dependent cargo sorting at endosomes[131], protein secretion from the *trans*-Golgi network[132] and mitosis[149]. Thus, perturbing clathrin (for example, through clathrin heavy chain RNA interference (RNAi)-mediated depletion[26] or by sequestering it with the assembly protein 180 (AP180) carboxyl terminus mutant[22]) will have consequences far beyond its role in endocytosis. For example, the plasma membrane levels of a receptor can be decreased by clathrin RNAi as a consequence of its defective Golgi export or endosomal recycling induced by clathrin depletion[131,132], leading to an indirect — and misleading — decreased internalization of its ligand. Moreover, because clathrin is also present on endosomes, live-cell imaging (even through total internal reflection fluorescence microscopy) of fluorescently labelled clathrin should be interpreted with caution, as not all signals recorded at (or near) the plasma membrane will be from clathrin-coated pits: a significant fraction could be from endosomes that are close to the plasma membrane[160].

Dynamin-dependent and clathrin-mediated endocytosis are not synonymous
Although it has been established that some clathrin-independent, dynamin-dependent endocytic pathways exist[161], many studies still use dynamin inhibition as evidence of clathrin-mediated endocytosis of cargo. Dynamin depletion (through RNAi), perturbation (using Lys44Ala[41] or Lys535Ala[162] mutants) or inhibition (using dynasore[42], dynole-34-2 (Children's Medical Research Institute/Newcastle Innovation)[163] or dyngo 4a (Children's Medical Research Institute/Newcastle Innovation)[164]) blocks endocytic processes far beyond clathrin-mediated endocytosis. Dynamin inhibition, although a powerful way to inhibit a large portion of cellular endocytosis, must therefore be complemented with more specific means of perturbation (see BOX 3) to assess whether a receptor or cargo enters cells specifically through clathrin-mediated endocytosis.

AP2 is the core plasma membrane adaptor for clathrin in metazoans
Although this remains controversial, there is so far no evidence for clathrin-coated pit formation at the plasma membrane in the absence of adaptor protein 2 (AP2). The arguments for the crucial role of AP2 in clathrin-mediated endocytosis are: its central location at the heart of the clathrin endocytic interactome (FIG. 2b); its requirement for the uptake of the best-characterized clathrin-dependent cargoes, transferrin, epidermal growth factor and low-density lipoprotein [26,27,102]; and the absence of direct evidence for the formation of clathrin-coated pits at plasma membranes that lack AP2 (REFS 26,27). Extensive depletion of AP2 is absolutely required for blocking the formation of clathrin-coated vesicles, and even after over 90% depletion by RNAi, AP2 can still be observed in the few clathrin-coated pits that are still forming[27]. We therefore believe that AP2 recruitment and AP2 depletion (through AP2 RNAi) should be part of the procedure for assessing whether a cargo enters cells through clathrin-mediated endocytosis (see BOX 3).

crucial role for AP2 in clathrin-coated vesicle formation at the plasma membrane (BOX 1).

Clathrin coat assembly. As cargo is selected and bound by AP2 or by cargo-specific adaptor proteins, the clathrin coat has to be assembled. Clathrin triskelia are recruited directly from the cytosol to sites of adaptor concentration on the membrane to help organize the forming coated vesicle. Clathrin is recruited to the plasma membrane by AP2 and also by accessory adaptor proteins (FIG. 2a,b). In the absence of clathrin, AP2 is recruited to the plasma membrane and forms puncta that colocalize with the nucleation complex, but the patch cannot mature[10] (FIG. 2c). Polymerization of clathrin results in stabilization of curvature and displacement to the edge of the forming vesicle of some cargo accessory adaptor proteins and curvature effectors, such as EPS15 and epsin[28,29], where they are likely to function most effectively. It had been assumed that clathrin polymerization can mediate membrane bending as the coated pit invaginates[30]. However, because clathrin binds to the flexible region of most adaptor proteins (AP2, AP180 and epsin), the potential force generated by polymerization would be inefficiently transmitted to deform the plasma membrane. Instead, direct membrane interactions of curvature effectors are thought to sculpt the vesicle (BOX 2). In some cell types, a substantial pool of clathrin can also be found as flat lattices (where triskelia are arranged as hexagons only)[31]. These lattices are found mostly on the cytoplasmic side of the adherent membrane surface and have a slower turnover than clathrin-coated pits[29].

Vesicle scission. Clathrin-coated vesicle budding depends on the mechanochemical enzyme dynamin[32]. It is recruited by BAR domain-containing proteins[33–35], which have a preference for the curvature of the vesicle neck and are likely to help form the neck (FIG. 2a). Examples of these proteins are amphiphysin, endophilin and sorting nexin 9 (SNX9), which have SRC homology 3 (SH3) domains that bind the Pro-rich domain of dynamin (FIG. 2b; TABLE 1). Polymerization around the neck of the nascent vesicle favours GTP hydrolysis and consequent membrane fission[36,37]. The precise mechanism is not clear, but the protein undergoes a GTP hydrolysis-dependent conformational change that probably helps to mediate scission[37–40]. Preventing the recruitment of dynamin, or inhibiting its activity, arrests vesicle formation at the stage of clathrin coat formation or vesicle scission[32,41,42]. Dynamin is also found in many other vesicle-budding pathways, in which it is recruited by a different subset of interaction partners (BOX 1).

Uncoating and clathrin component recycling. Once detached from the parent membrane, the clathrin coat is disassembled from its lattice arrangement back to triskelia by the ATPase heat shock cognate 70 (HSC70) and its cofactor, auxilin (or cyclin G-associated kinase (GAK) in non-neuronal tissues)[43,44] (FIG. 2a,b), allowing the detached and uncoated vesicle to travel to and fuse with its target endosome. Auxilin is recruited after clathrin-coated vesicle budding[45,46] by binding to the terminal domains and ankles of clathrin triskelia[47,48], and it localizes under the 'hub' of a neighbouring triskelium[48]. There, auxilin recruits HSC70 to a specific motif located at the foot of the tripod below the clathrin hub[49,50], from which the uncoating reaction is initiated. One auxilin and three or fewer HSC70 molecules are needed per triskelia to get maximum disassembly *in vitro*[51,52]. It is important to remember that, when clathrin-coated vesicle scission takes place, it is unlikely that the clathrin cage is completed across the zone where the neck was attached, thus leaving a defect in the clathrin cage that allows the uncoating apparatus to start the uncoating process with ease. We predict that this is why uncoating takes place only after vesicle scission, as this is the only point when a defect becomes apparent. Changes in the phosphoinositide composition of clathrin-coated vesicles mediated by the phosphatase synaptojanin are

Table 1 | **Glossary of proteins involved in clathrin-mediated endocytosis**

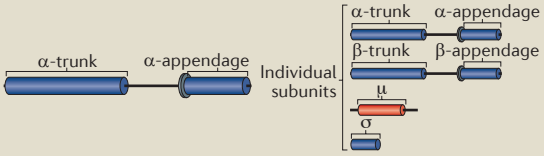
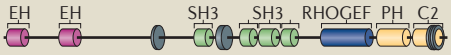
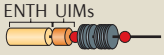
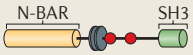
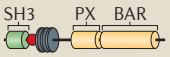
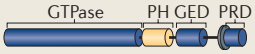
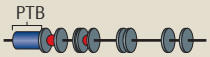
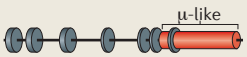
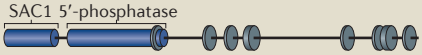
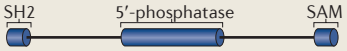
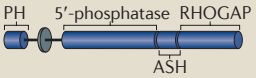
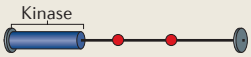
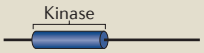
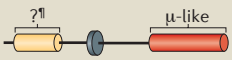
Protein	Human genes	Function	Domain architecture*
Core components			
Clathrin	*CLTA*[‡], *CLTB*, *CLTC*	Self-polymerizing protein composed of three heavy and three light chains that form the clathrin triskelion, which can polymerize into flat lattices or cages	
FCHO	*FCHO1*, *FCHO2*[‡]	F-BAR domain-containing proteins that nucleate clathrin-coated pits and generate the initial membrane curvature	
AP2	*AP2A1*[‡], *AP2A2*, *AP2B1*, *AP2M1*, *AP2S1*	A heterotetrameric adaptor complex (α-, β2, μ2 and σ2 subunits) that links membrane cargo to clathrin and accessory proteins	
EPS15–EPS15R	*EPS15*[‡], *EPS15R*	AP2 clustering and scaffolding proteins	
Intersectin	*ITSN1*[‡], *ITSN2*	Scafolding protein linking various components of the clathrin machinery	
AP180, CALM[§]	*SNAP91*[‡], *PICALM*	ANTH domain-containing PtdIns(4,5)P_2-binding protein that binds AP2 and clathrin and is thought to regulate vesicle size	
Epsin	*EPN1*[‡], *EPN2*	ENTH domain-containing membrane-bending protein that is a cargo-specific adaptor for monoubiquitylated receptors	
Amphiphysin	*AMPH1*[‡], *BIN1*	N-BAR domain-containing protein that bends the membrane and recruits dynamin to clathrin-coated pits	
SNX9	*SNX9*	BAR domain-containing protein that binds AP2 and dynamin	
Dynamin	*DNM1*[‡], *DNM2*, *DNM3*	Self-polymerizing mechanoenzyme that triggers vesicle scission upon GTP hydrolysis	
Auxilin, GAK[‖]	*DNAJC6*[‡], *GAK*	J domain-containing protein that recruits HSC70 to clathrin cages for uncoating	
HSC70	*HSPA8*	ATPase triggering uncoating of clathrin cages	
Cargo-specific adaptors			
ARH	*LDLRAP1*	Recruits LDLR to AP2	
DAB2	*DAB2*	Recruits megalin and LDLR to AP2	
Stonin	*STON1*, *STON2*[‡]	Recruits synaptotagmin to AP2	
HRB	*AGFG1*	Recruits the SNARE protein VAMP7 to AP2	
NECAP	*NECAP1*[‡], *NECAP2*	Potential cargo-specific adaptor	
Numb	*NUMB*	Recruits Notch to AP2	
β-arrestin	*ARRB1*, *ARRB2*[‡]	Recruits GPCRs to AP2 and clathrin	

Table 1 cont. | **Glossary of proteins involved in clathrin-mediated endocytosis**

Protein	Human genes	Function	Domain architecture*
Inositol 5-phosphatases			
Synaptojanin	*SYNJ1*‡, *SYNJ2*	Lipid phosphatase recruited to clathrin-coated pits by AP2 and endophilin	
SHIP2	*INPP1L*	Lipid phosphatase recruited to clathrin-coated pits by intersectins	
OCRL	*OCRL1*	Lipid phosphatase recruited to mature clathrin-coated pits by AP2 and clathrin	
Kinases			
AAK1	*AAK1*	Phosphorylates the μ-chain of AP2	
CVAK104	*SCYL2*	Phosphorylates the β2 subunit of AP2	
DYRK1A	*DYRK1A*	Phosphorylates several proteins involved in clathrin-mediated endocytosis	
Actin nucleation at clathrin-coated vesicles			
HIP1–HIP1R	*HIP1*‡, *HIP1R*	ANTH domain-containing proteins that bind clathrin, actin, AP2 (HIP1 only) and cortactin (HIR1R only)	
Cortactin	*CTTN*	Recruits actin polymerization machinery to budding clathrin-coated vesicle through dynamin and HIP1R	
Other proteins potentially involved in clathrin-mediated endocytosis			
Endophilin	*SH3GL1*‡, *SH3GL2*, *SH3GL3*	N-BAR domain-containing protein that bends the membrane and recruits dynamin and synaptojanin	
SGIP1	*SGIP1*	Membrane-tubulating protein containing a μ-homology domain	
TTP	*SH3BP4*	Controls TfR recruitment to clathrin-coated pits	

AAK1, AP2-associated kinase 1; AGFG1, ARFGAP with FG repeats 1; ANTH, AP180 amino-terminal homology domain; AP2, adaptor protein 2; ARH, autosomal recessive hypercholesterolaemia; ASH, ASPM1–SPD2–hydin; CALM, clathrin assembly lymphoid myeloid leukaemia; CLT, clathrin light chain; CVAK104, coated vesicle associated kinase of 104 kDa; DAB2, Disabled homologue 2; DYRK1A, dual-specificity Tyr phosphorylation-regulated kinase; EH, EPS15 homology; ENTH, epsin N-terminal homology domain; EPS15, EGFR pathway substrate 15; EPS15R, EPS15-related; FCHO, FCH domain only; GAK, cyclin G-associated kinase; GAP, GTPase-activating protein; GED, GTPase effector domain; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; HIP1, huntingtin-interacting protein 1; HIP1R, HIP1-related; HSC70, heat shock cognate 70; LDLR, low-density lipoprotein receptor; NECAP, adaptin ear-binding coat-associated protein; OCRL, oculocerebrorenal syndrome of Lowe; PH, pleckstrin homology; PICALM, phosphatidylinositol-binding CALM; PRD, Pro-rich domain; PTB, phosphotyrosine binding; PTEN, phosphatase and tensin homologue; PX, phox homology; SAC1, suppressor of actin; SAM, sterile α-motif; SGIP1, SH3-containing GRB2-like 3-interacting protein 1; SH, SRC homology; SHIP2, SH2 domain-containing inositol phosphatase 2; SNAP91, synaptosomal-associated protein 91 kDa homologue; SNX9, sorting nexin 9; TfR, transferrin receptor; TTP, TfR trafficking protein; UIMs, ubiquitin-interacting motifs; VAMP7, vesicle-associated membrane protein 7. *Clathrin-binding motifs are denoted by a red dot. ‡Denotes the proteins for which the structure is depicted in the domain architecture column. §AP180 is the brain-specific protein, CALM is the ubiquitous one. ‖Auxilin is the brain-specific protein, GAK is the ubiquitous one. ¶Lipid binding module of undefined character.

required for uncoating[53], but whether synaptojanin acts by facilitating auxilin recruitment[45] or another mechanism is not yet clear. Uncoating releases the clathrin machinery back into the cytoplasm to be recruited and reused in another round of clathrin-coated vesicle formation.

Pathway modularity

At the heart of clathrin-coated vesicle formation is a flexible network of interactions, which can be thought of as being made up of different modules[54,55]. As vesicle formation progresses, a corresponding protein interaction network drives these changes (FIG. 2b). This network carries out initiation, cargo selection, membrane invagination, coat assembly, membrane scission and uncoating of the newly formed vesicle[54,55].

Global modularity. Although complex, the interactions between proteins involved in clathrin-coated vesicle formation are not random and can be grouped into five modules made up of proteins interacting to carry out the five stages of clathrin vesicle formation. These protein interactions are thought to surround a hub (or organizing) protein (FIG. 2b) with its associated accessory proteins[54]. The transition between the five modules is probably controlled at the different stages

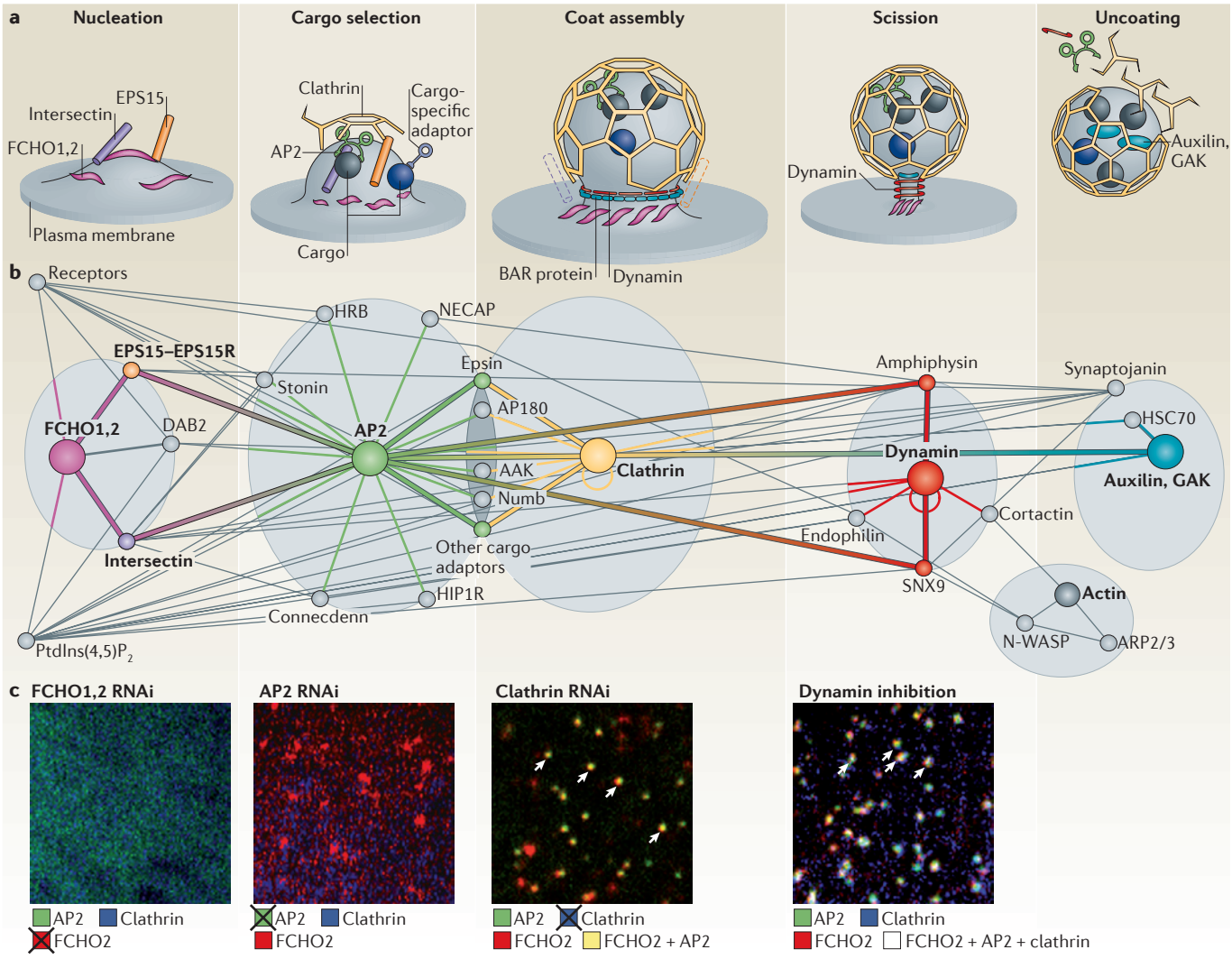
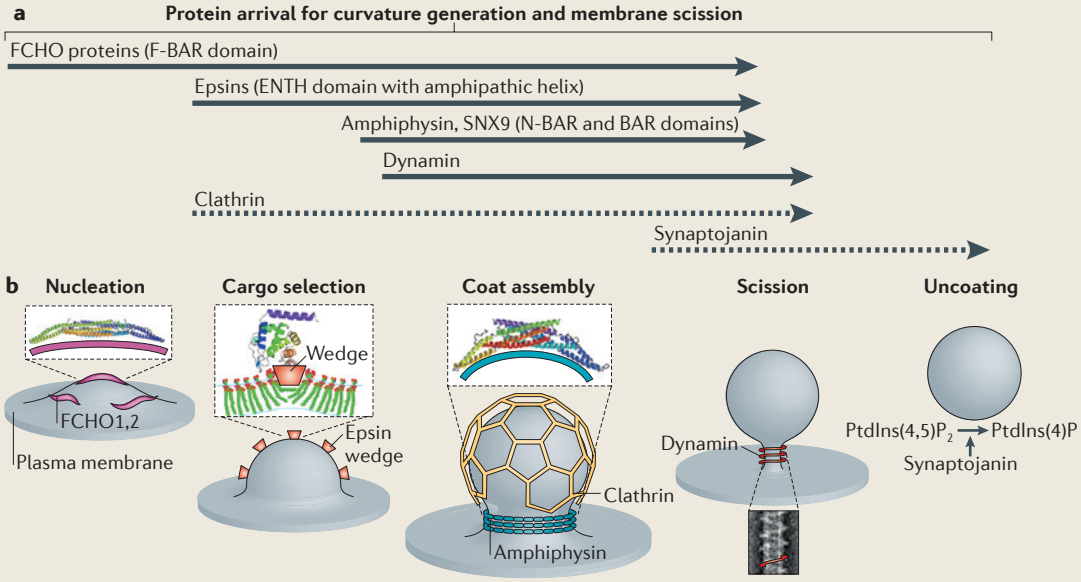


Figure 2 | **The clathrin-coated vesicle cycle. a** | The proposed five steps of clathrin-coated vesicle formation. Nucleation: FCH domain only (FCHO) proteins bind phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2)-rich zones of the plasma membrane and recruit EPS15–EPS15R (EGFR pathway substrate 15–EPS15-related) and intersectins to initiate clathrin-coated pit formation by recruiting adaptor protein 2 (AP2). Cargo selection: AP2 recruits several classes of receptors directly through its μ-subunit and σ-subunit. Cargo-specific adaptors (for example, stonin, HRB and Numb) bind to AP2 appendage domains and recruit specific receptors to the AP2 hub. Coat assembly: clathrin triskelia are recruited by the AP2 hub and polymerize in hexagons and pentagons to form the clathrin coat around the nascent pit. Scission: the GTPase dynamin is recruited at the neck of the forming vesicle by BAR domain-containing proteins, where it self-polymerizes and, upon GTP hydrolysis, induces membrane scission. The actin machinery module can be added at this stage for actin polymerization at the neck of the pit, which can aid in vesicle production (not shown). Uncoating: auxilin or cyclin G-associated kinase (GAK) recruit the ATPase heat shock cognate 70 (HSC70) to disassemble the clathrin coat and produce an endocytic vesicle containing the cargo molecules. Synaptojanin probably facilitates this by releasing adaptor proteins from the vesicle membrane through its PtdIns lipid phosphatase activity. The components of the clathrin machinery are then freed and become available for another round of clathrin-coated vesicle formation. **b** | The clathrin network. The protein–protein interactions underlying the different stages of vesicle progression are shown. Major hubs are obvious because of their central location in the network and the large number of interacting molecules. They are essential for pathway progression and are denoted by the central coloured circles. Possible pathways of progression between hubs are shown with thicker lines. **c** | Effects of depletion of different components of the clathrin machinery. Epithelial cells expressing FCHO2 tagged with red fluorescent protein (RFP), AP2 tagged on the σ2 subunit with enhanced green fluorescent protein (σ2–EGFP) and clathrin tagged with blue fluorescent protein (BFP) show arrests at each successive stage in clathrin-coated pit formation after perturbation of major hubs. FCHO protein depletion through RNA interference (RNAi) inhibits AP2 and clathrin recruitment to the plasma membrane (and both remain diffuse and cytosolic). AP2 RNAi abrogates clathrin recruitment to the plasma membrane but not FCHO2 initial focus formation (dense red spots). Clathrin RNAi blocks maturation during cargo selection, when FCHO2 and AP2 are clustered (yellow dots, arrows). Dynamin inhibition (by dynasore) blocks clathrin-coated pits at a stage just before membrane scission (white dots, arrows). AAK, AP2-associated protein kinase 1; ARP2/3, actin-related protein 2/3; DAB2, Disabled homologue 2; HIP1R, HIP1-related; N-WASP, neural Wiskott–Aldrich syndrome protein; NECAP, adaptin ear-binding coat-associated protein; SNX9, sorting nexin 9.

Box 2 | **Curvature and clathrin-coated vesicle formation**



Many different curvature effectors have been found to work in clathrin-mediated endocytosis to drive a piece of flat membrane into a highly curved cargo-loaded vesicle. This range of effectors may reflect the different degrees or angles of curvature to be obtained or the evolutionary adaptation of different cargo adaptors as curvature adaptors (see the figure, part **a**).

Membranes can be bent by forces acting in the membrane or on the membrane (see the figure, part **b**). Shallow insertions within the membrane can work like wedges and lead to membrane curvature. For a wedge to work effectively, the insertion must occupy a greater volume at the membrane surface than at the membrane midline. An amphipathic helix, such as the one in the epsin N-terminal homology (ENTH) domain[22], inserted like a wedge in the plane of the membrane, results in lipid molecules tilting and splaying to accommodate the helix; this results in positive curvature towards the insertion[165].

Proteins working on the membrane can affect its curvature by having a significant contact surface and affinity with the membrane, such that the membrane is induced to follow the shape and curvature of the interacting proteins (or indeed a protein oligomer)[165]. This can lead to rather complex shapes, but works for local and high-curvature intermediates. The main examples of these types of motifs in clathrin-mediated endocytosis are BAR, N-BAR and F-BAR domains[166–168]. Clathrin forms a lattice (involving hexagons and pentagons) around a forming vesicle bud and thus stabilizes membrane curvature. It is unlikely to be the driving force (indicated by dotted arrow in figure part **a**), but, given that it binds to curvature effectors that in turn promote its polymerization, it probably also has a role in curvature formation.

Dynamin is the candidate protein for breaking the continuity of the nascent vesicle with the plasma membrane and the concomitant resealing of the separated membranes. This is the most complex membrane remodelling that takes place during vesicle formation. Although not well understood, it requires energy in the form of GTP hydrolysis[37,38] and involves both scaffolding of curvature and membrane insertions[169,170].

Synaptojanin, the lipid phosphatase that dephosphorylates inositol headgroups, works in a curvature-sensitive manner, so its activity is much higher for the nascent vesicle than for the plasma membrane[171]. This will likely aid the release of adaptor protein 2 (AP2), epsin and AP180, all of which bind to membranes in a phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$)-dependent manner.

FCHO, FCH domain only; SNX9, sorting nexin 9.

of clathrin-coated vesicle formation, as vesicle formation is blocked by disruption at each respective stage (BOX 3; FIG. 2c). Some modules are interchangeable; for example, AP1 or AP3 can be substituted for AP2 to form clathrin-coated vesicles from different cellular compartments (for example, endosomes and the *trans*-Golgi network (TGN)). Other modules have similar functions in the various pathways; for example, the clathrin module can be used in vesicle budding from other cellular compartments, and the dynamin module can be used in many different vesicle scission events. The specificity or efficiency of vesicle formation can be changed by adding accessory modules to the core modules, without compromising the production of vesicles. For example, alternative adaptor proteins can be added at the plasma membrane that provide cargo specificity (such as autosomal recessive hypercholesterolaemia (ARH), Numb, stonin, HRB and β-arrestin), or connections to the actin machinery can be activated to aid vesicle budding. These modules are accessory, as they show differential representation in different species (discussed below) and variable distribution in different cell types, or even within an individual cell.

Global regulation of endocytosis through phosphorylation is well characterized in synapses (see below), where the initiation of clathrin-mediated endocytosis is

Synapses
Specialized junctions between cells that allow neurons to transmit chemical signals to other cells (neural or otherwise).

Box 3 | **Inhibiting clathrin-mediated endocytosis**

Traditionally, clathrin-mediated endocytosis has been inhibited by the use of monodansylcadaverine (MDC)[172], potassium depletion[173], phenylarside oxide (PAO)[174], cytosolic acidification[175], hypertonic shock (sucrose)[176] or chlorpromazine[177]. However, because of their pleiotropic effects, the overexpression of protein interaction domains taken from endocytic proteins (for example, the carboxyl terminus of adaptor protein 180 (AP180)[22] or EGFR pathway substrate 15 (EPS15) with deletions of the second and the third EPS15 homology (EH) domains ($EPS15^{\Delta 95/295}$))[178] has become a method of choice, although these may not be specific either if they titrate out components that are used in other trafficking pathways, such as clathrin (see BOX 1). Thus, for specificity, it is advisable to target interaction domains that are specific for nucleation and clathrin-coated pit formation (FIG. 2). Overexpression of the C-terminal-tagged FCH domain only (FCHO) protein would be a good specific inhibitor for clathrin-mediated endocytosis. Another popular method is RNA interference (RNAi) of clathrin heavy chain and of the α-subunit or μ2-subunit of AP2.

Different considerations need to be taken when using the two approaches. When using RNAi, one should deplete a hub (FCHO proteins[10] or the α-subunit or μ2-subunit of AP2 (REFS 26,27)) rather than an accessory protein, as these are central organizing components. By contrast, overexpression of accessory proteins may be better in overexpression studies, as it is easy to distort the network by pulling it towards a non-functional point. For an overexpression strategy to work on hubs (which tend to be virtual polymers) one needs to make sure that the inhibitory domain is incorporated into the polymer. Both approaches require transfection and a long incubation period (>16 hours (mutant); 48–72 hours (RNAi)) to be efficient and to produce a steady-state effect, and during this time compensatory events may occur.

Strategies using small molecule inhibitors and protein complementation are in development to acutely perturb the pathway. Acute inhibitors of dynamin[42,163,164] exist and are already widely used, although precautions are required as dynamin functions in several pathways beyond clathrin-mediated endocytosis (BOX 1). 'Derouting' AP2 to a location where it is not normally present (such as mitochondria) using small molecule-mediated heterodimerization is a fast way to relocalize clathrin and inhibit clathrin-mediated endocytosis[179].

With the emerging use of chemical biology, we can look forward to the development of inhibitors specific to clathrin-mediated endocytosis, which will allow more acute inhibition and thus more accurate determination of the importance of the pathway. Perhaps what we now require is not new pathway inhibitors but precise inhibitors of specific receptor recruitment into coated pits, so that we can probe endocytosis with minimal side effects on the endocytosis of other receptors at the same time.

triggered by calcineurin-mediated dephosphorylation of many endocytic components, which are then rephosphorylated by cyclin-dependent kinase 5 (CDK5)[56,57]. We believe that this same regulation, mediated by a different phosphatase, may apply to clathrin-mediated endocytosis in other cell types. These types of regulation remain largely unexplored.

Modularity in cargo selection. The presence of different cargo adaptor proteins, including the ubiquitous protein AP2 and many accessory cargo adaptor proteins, ensures that specific receptors are recruited to the protein interaction network. For example, ARH is a specific adaptor for low-density lipoprotein receptors (LDLRs)[58,59], Numb binds Notch[60], stonin binds synaptotagmin[17], HRB binds SNARE proteins[19], and β-arrestin and Dishevelled bind G protein-coupled receptors (GPCRs)[18,61]. These accessory cargo adaptor proteins bind directly to the core AP2 adaptor appendage domains and therefore do not compete with direct cargo binding. They may be expressed by distinct cell types to confer tissue specificity; for example, stonins are found in neurons and thereby help to recruit the calcium sensor synaptotagmin specifically into synaptic vesicles. Other tissues, such as the liver, have cargo adaptor proteins, such as ARH, that bind to LDLR to allow its incorporation into clathrin-coated vesicles. Furthermore, different accessory adaptor proteins can be expressed in different tissues, thus allowing a whole range of proteins to be recruited into a clathrin-coated vesicle. In addition to conferring specificity, the presence of distinct cargo adaptor proteins ensures that, when one receptor (for example, TfR) is present in high levels on the membrane, this does not block the uptake of non-cognate cargo (in this case, EGF or LDL[62,63]). Therefore, even if a receptor is present on the membrane with low representation, it can still be endocytosed.

Functional perturbation (mutations or decreased expression) of a cargo-specific adaptor protein is sufficient to specifically decrease the cellular uptake of its cognate receptor without affecting the formation of clathrin-coated vesicles and the internalization of other cargoes. For example, perturbation of the adaptor protein ARH specifically affects the recruitment of LDLRs to clathrin-coated pits[58,59]. Consistently, increased expression of cargo-specific adaptor proteins increases the internalization of their cognate receptors and cargoes[61,64], and some cargo-specific effects on clathrin-mediated endocytosis have been reported upon overexpression of artificial chimeric receptors[65,66].

It therefore seems that cargo-specific adaptor proteins work like add-on modules, giving extra functionality that is adapted to the respective cellular need. It is possible that artificial manipulation of cargo-specific adaptor proteins could potentially be used for increasing targeted drug delivery for specific diseases.

Modularity in actin recruitment. Actin is not necessary for the endocytosis of transferrin (the classical ligand used to study clathrin-mediated endocytosis) by clathrin-mediated endocytosis in several cell types[67,68], and its disruption does not inhibit early hub progression. Therefore, it is not regarded as a core component of the network. In the situations in which actin is reported to function, it is a late component that is recruited close to the time of membrane scission[46]; thus, it is likely to help in the budding of some vesicle types. Actin joins the clathrin network following the recruitment of the actin-nucleating complex actin-related protein 2/3 (ARP2/3) to the budding vesicle. This can be mediated by cortactin or by neural Wiskott–Aldrich syndrome protein (N-WASP), which is itself recruited by the SH3 domains of BAR domain-containing proteins (TABLE 1). The actin module is not unique to the clathrin pathway and functions in other contexts. During clathrin-mediated endocytosis, it may offer additional functionality in the form of energy for vesicle budding and scission. It is interesting that there is a correlation between the time taken for the formation of clathrin-coated vesicles and the detection of actin, with the longest pits having the most actin recruited[69]. It is thus possible that an actin polymerization module is added to the network when there is a need to produce force, because of either the size of the cargo or the rigidity of the membrane. Consistent with this, actin is crucial

SNARE proteins
(Soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor proteins). Members of a family of membrane-tethered coiled-coil proteins that regulate fusion reactions and target specificity. On the basis of their localization, they can be divided into vesicle membrane SNAREs (v-SNAREs) and target membrane SNAREs (t-SNAREs).

G protein-coupled receptors
(GPCRs; also known as seven transmembrane domain receptors). The largest family of cell surface receptors (>800 members) that sense molecules outside the cell and activate signal transduction pathways.

for clathrin-mediated endocytosis in yeast[54], as yeast cells have a rigid wall and an internal pressure against which a higher force has to be applied to invaginate the membrane compared with mammalian cells[70]. It will be interesting to assess the role of actin in clathrin-coated vesicle formation in plants, as they also have a rigid wall and a high internal pressure. Actin polymerization is also required during uptake of large cargoes, such as viruses and bacteria, in mammalian cells[69,71] (see below). In this case, the clathrin lattice may serve as a signalling platform for actin polymerization, which may thus make it indispensible for cellular entry of these very large cargoes.

Species variation

The clathrin heavy chain is conserved across all eukaryotic genomes, and clathrin-coated vesicles have been observed or isolated from many unicellular and multicellular eukaryotes[4,72–75]. However, differences between species have been observed, such as the dependency of endocytosis on clathrin, adaptors and actin, and the kind of ligand internalized.

The size of clathrin-coated pits varies. When clathrin-coated pits from different species are observed to scale, they seem to differ widely in size (FIG. 3a). The size of clathrin-coated vesicles depends on the size of its cargo[76], with an observed upper limit of about 200 nm external diameter, as in the case of virus uptake[69] (FIG. 3a). Larger cargoes, such as latex beads (mimicking phagocytosis) and bacteria, do not seem to be taken within single clathrin-coated vesicles (FIG. 3b). Yeasts (for example, *Saccharomyces cerevisiae*) and plants (for example, *Arabidopsis thaliana*) have very small coated pits of ~35–60 nm in diameter (the exterior diameter comprising the coat)[77,78], which is considerably smaller than the ones found in mouse fibroblasts[79] or chicken oocytes[80] (which are ~150 nm in diameter). This may be because plant cells and yeasts have rigid walls, so having to counteract the internal pressure to deform the plasma membrane might limit the size of the clathrin-coated pits. Brain-derived clathrin-coated vesicles have external diameters of ~70–90 nm[81], with the internal vesicles having diameters of ~34–42 nm[81,82]. Thus, the fact that the clathrin-coated vesicles in yeasts and plants have diameters of ~35–60 nm indicates that their internal vesicles would measure ~15–25 nm in diameter; this is probably too small to package large cargoes or a large number of small cargoes. Indeed, human LDL bound to its receptor has a diameter of 25 nm (FIG. 3c) and would not fit into a yeast or a plant clathrin-coated pit. TfR and EGFR bound to their cognate cargoes occupy diameters of 11 nm and 13 nm, respectively, and could not be loaded at more than one or two copies at a time into such small clathrin-coated pits (FIG. 3c). Only very small receptors, such as GPCRs, which have much smaller luminal occupancy (~4 nm in diameter), could fit into yeast or plant clathrin-coated pits, but the internalization of such receptors functions in signal transduction regulation and not in ligand uptake (FIG. 3c). This indicates that the primary role of clathrin-mediated endocytosis may not be internalization of extracellular ligands in yeast and plants (see below for other arguments).

The size of clathrin-coated pits also varies between different cell types within the same species. For example, clathrin-coated pits in rat and mouse brains are ~70–90 nm in diameter, which is also comparable to the pit size in lamprey synapses (FIG. 3a). However, they are significantly smaller that those generally observed in mouse or human epithelial cells, which are ~120–150 nm in diameter[79]. This may be because the brain vesicles do not internalize large extracellular cargo but simply retrieve the synaptic vesicle components.

Clathrin-mediated endocytosis is not essential in single-cell organisms. Clathrin perturbations are lethal in multicellular organisms, such as *Drosophila melanogaster*, *Caenorhabditis elegans* and *Mus musculus*[83,84]. However, unicellular organisms, such as yeasts, amoebae (for example, *Dictyostelium discoideum* during its vegetative state) and protozoa (for example, *Trypanosoma brucei*), are viable but weak following clathrin ablation, and their main defects seem to come from inhibition of intracellular trafficking (such as from the Golgi to the vacuole) instead of endocytic defects[85–87]. Consistent with this, the core components of clathrin-mediated endocytosis (AP2, intersectin, EPS15–EPS15-related (EPS15R), dynamin, synaptojanin and auxilin) are also either not essential or do not have homologues in unicellular organisms[78,86,88,89], whereas genetic ablation of any of these components in flies, worms and mice is embryonic lethal[34,53,90–94]. Furthermore, there is a paucity of cargo-specific adaptor proteins in unicellular organisms, whereas in higher eukaryotes knockouts of individual cargo-specific adaptor proteins or accessory proteins, such as stonin, Disabled homologue 2 (DAB2) or AP180, have mild to severe phenotypes but all are still capable of endocytosis[95–99]. This suggests that these proteins serve more specialized functions in higher eukaryotes, highlighting the importance of maintaining clathrin-mediated endocytosis in higher organisms.

Clathrin might function in receptor-mediated endocytosis only in higher organisms. Clathrin mutations in unicellular organisms, such as protozoa and amoebae, block GPI-anchored protein and global plasma membrane turnover as well as fluid-phase uptake[86,100], processes that are largely independent of clathrin in higher organisms (reviewed in REF. 1). This, together with the scarcity or absence of clathrin adaptors in these organisms[86], suggests that clathrin-mediated endocytosis might serve as a generic non-specialized endocytic portal in lower organisms. Consistent with this, only a few receptors have been shown to enter cells through clathrin- and actin-mediated endocytosis in yeast, and none of these seems to be totally dependent on clathrin. For example, clathrin mutants still internalize α-factor and its receptor, Ste2, albeit at slower rates (~40–60%) than wild-type cells[88,101]. By contrast, in metazoans many receptors (for example TfR and LDLR) are highly (>80%) dependent on clathrin-mediated endocytosis to enter cells[26,27,102] (it is important to note that differences in the assays used in yeast and mammalian cells might influence the previously

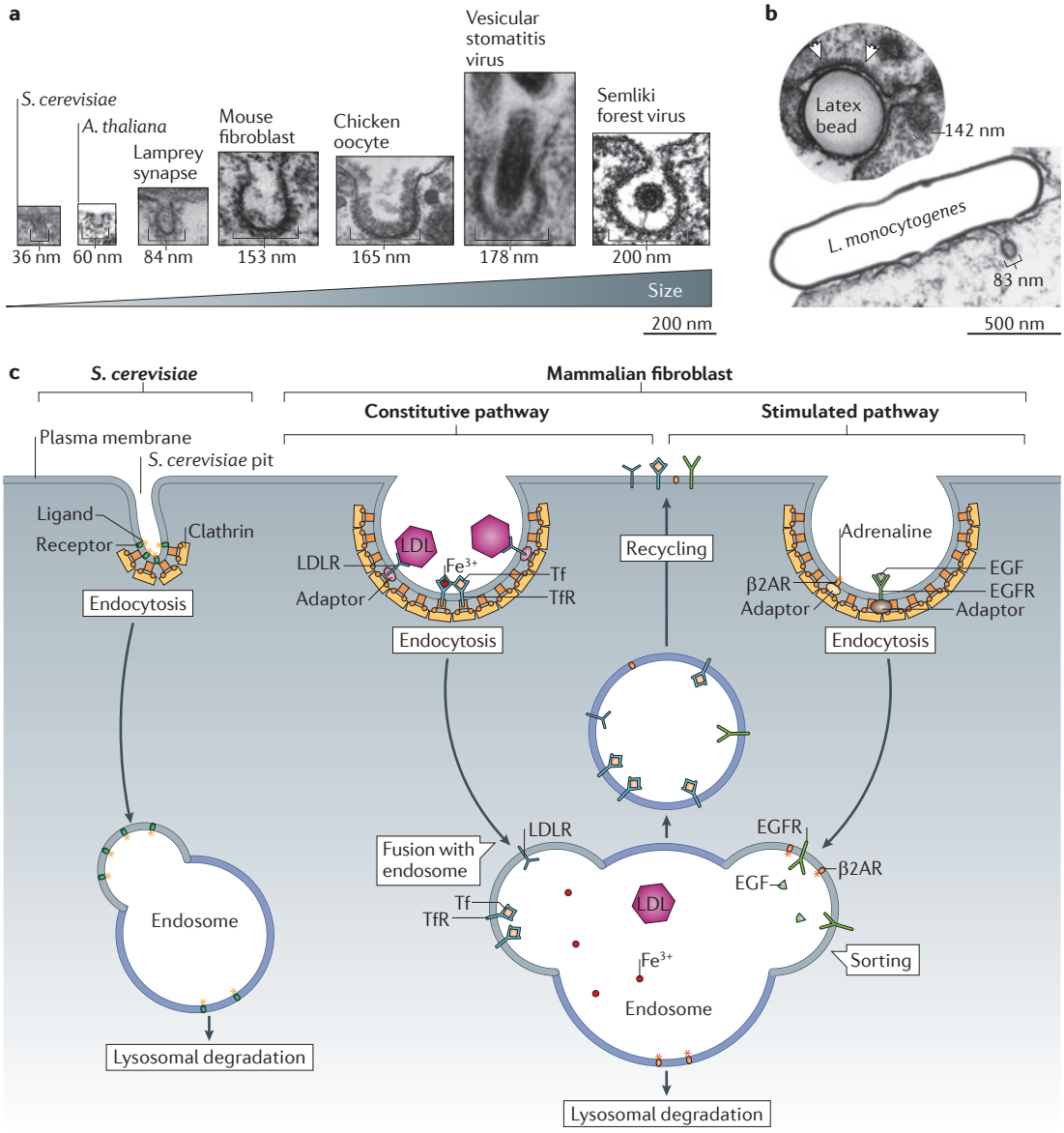
Phagocytosis
A specific form of endocytosis involving the internalization of solid particles, such as bacteria.

Vacuole
A membrane-bound organelle that is present in all plant and fungal cells, as well as in the cells of some other organisms. It is the equivalent to the lysosomes in other organisms.

GPI-anchored protein
A protein anchored by glycosylphosphatidylinositol (GPI) in the secretory pathway to reach the extracellular leaflet of the plasma membrane.

Fluid-phase uptake
(Also called macropinocytosis). Endocytosis of large (0.5–5 mm diameter) vesicles derived from ruffling of the plasma membrane, taking up extracellular fluid in a nonspecific manner.

α-factor
A yeast mating pheromone that is recognized by the receptor Ste2.



◀ Figure 3 | **Species variations and physiological functions of clathrin-mediated endocytosis. a** | Electron micrographs of clathrin-coated pits in various organisms and with diverse cargoes, from small (*Saccharomyces cerevisiae*) to large (Semliki forest virus). Yeasts (for example, *S. cerevisiae*) and plants (for example, *Arabidopsis thaliana*) have the smallest clathrin-coated pits. Within the same species, clathrin-coated pits seem to be smaller in neurons (in synapses) than in fibroblasts. **b** | Electron micrographs of clathrin-mediated endocytosis of a latex bead and *Listeria monocytogenes*. Flat clathrin lattices (arrowheads) as well as clathrin-coated pits are seen surrounding some of the latex bead-containing phagosome. These cargoes are too large to be accommodated in single clathrin-coated pits. **c** | Cargoes of various sizes can be endocytosed by clathrin-coated pits in a constitutive or stimulated manner. Comparisons of cargo in fibroblast clathrin-coated pits (middle and right; ~100 nm internal vesicle diameter) emphasize the constraints placed on endocytosis by smaller pits in *S. cerevisiae* (left; ~10 nm internal diameter). Receptors and ligands of various sizes (drawn here to scale with respect to the pits) can fit inside fibroblast clathrin-coated pits, whereas only very small receptors, such as G protein coupled receptors (GPCRs), can be accommodated inside a *S. cerevisiae* clathrin-coated structure (left). Some receptors, such as transferrin receptor (TfR) and low-density lipoprotein receptor (LDLR), do not require bound cargo for internalization by clathrin-mediated endocytosis (via the constitutive pathway), whereas others, such as epidermal growth factor receptor (EGFR) and β2 adrenergic receptor (β2AR) are endocytosed upon ligand binding. Endocytic motifs present in the cytoplasmic tails of constitutively endocytosed receptors are recognized by adaptor protein 2 (AP2) or cargo-specific adaptors and are then sorted into clathrin-coated pits for internalization into the cell. During stimulated endocytosis, ligand-induced receptor dimerization (EGFR) or deprotection (β2AR) (dimerization and deprotection not shown) allow receptors to bind AP2 or cargo-specific adaptors, thus facilitating their recruitment into clathrin-coated pits. In both cases, the low pH of endosomes induces the release of ligands (or, in the case of TfR, of the bound Fe^{3+} from transferrin (Tf)) when bound to receptors. Receptors can then be recycled back to the plasma membrane for another round of trafficking, or routed to lysosomes to be degraded (applies to ligand-bound receptors). **d** | Synaptic vesicle recycling. Action potentials and depolarization-induced Ca^{2+} influx triggers synaptic vesicle fusion and neurotransmitter release. Synaptic vesicle components (for example, SNAREs, synaptotagmin and neurotransmitter transporters) are sorted into clathrin-coated pits by binding AP2 and cargo-specific adaptors. Recycled synaptic vesicles are translocated to endosomes, where they are reloaded with neurotransmitters for another round of release. The image of the *S. cerevisiae* vesicle is courtesy of K. Ayscough, University of Sheffield, UK. The image of the *A. thaliana* vesicle is reproduced, with permission, from REF. 77 © (2007) Elsevier. The image of the lamprey synapse is reproduced, with permission, from REF. 180 © (2010) National Academy of Sciences. The image of the mouse fibroblast is reproduced, with permission, from REF. 79 © (1980) National Academy of Sciences. The image of the chicken oocyte is reproduced, with permission, from REF. 80 © (1979) The Company of Biologists. The image of the vesicular stomatitis virus vesicle is reproduced, with permission, from REF. 134. The image of the Semliki forest virus vesicle is courtesy of J. Kartenbeck and A. Helenius, Swiss Federal Institute of Technology Zürich, Switzerland. The image of the latex bead is reproduced, with permission, from REF. 181 © (1982) The Rockefeller University Press. The image of *L. monocytogenes* in reproduced, with permission, from REF. 182 © (1996) Elsevier.

mentioned results). Thus, clathrin-mediated endocytosis may have evolved to have a more specific role of selective internalization of plasma membrane receptors in higher organisms.

Physiological functions

The cellular uptake of plasma membrane receptor proteins mediated by clathrin is implicated in many key cellular functions. Clathrin-mediated endocytosis controls constitutive and stimulated internalization of many receptors with roles in cellular homeostasis, growth control, cell differentiation and synaptic transmission. The pathway is also exploited by toxins, viruses and bacteria to gain entry into cells.

Constitutive and stimulated internalization of receptors. The first described physiological function of clathrin-mediated endocytosis was to selectively internalize receptors carrying metabolites such as cholesterol or iron[103,104]. In metazoans, several receptor types are endocytosed constitutively (that is, with or without ligands bound) from the plasma membrane to endosomes, and a major portion of these is recycled back to the plasma membrane within minutes (FIG. 3c). TfR is the classic example of such a receptor and is used in many studies to assess clathrin-mediated endocytic activity, as its trafficking relies mainly on this pathway[26,105]. LDLR is also reported to traffic constitutively through clathrin-mediated endocytosis[103]. Many receptors, including receptor Tyr kinases (RTKs) (such as EGFR) and GPCRs (for example, β2 adrenergic receptor), undergo stimulated, or ligand-induced, endocytosis (FIG. 3c). Binding of the cognate ligands induces dimerization (in the case of RTKs) or a change in conformation (in the case of GPCRs) of the receptors, and this is necessary for their recruitment by adaptors to clathrin-coated pits. Once internalized, receptors are either freed from their ligands and recycled back to the cell surface or sorted to lysosome for degradation (FIG. 3c). All of these receptors rely on AP2 for recruitment to clathrin-coated pits, either directly (as with TfR[26] and EGFR[102]) or through cargo-specific adaptor proteins (as with LDLR, which requires DAB2 and ARH[59], and β2 adrenergic receptor, which requires β-arrestin[61]).

Signal transduction regulation. By influencing the surface protein composition of cells, clathrin-mediated endocytosis controls receptor signalling, responses to channel activation and transporter activity. Clathrin-mediated endocytosis controls the activity of many RTKs (for example, EGFR and insulin receptors) and GPCRs (for example, β2 adrenergic receptor and Frizzled), as well as the activity of synapses and of transporters (for example, amine and auxin transporters)[106–109]. The signalling pathways involved have wide consequences for major cellular functions, ranging from cell growth, division and differentiation to synaptic transmission, development, chemotaxis and immune responses (reviewed in REFS 106–109). The classical function of clathrin-mediated endocytosis in the regulation of signal transduction is to terminate the signal by physically removing activated receptors from the cell surface[106,108]. The internalization of ligand–receptor complexes into endosomes and then lysosomes may lead to their degradation, which results in termination of signalling (FIG. 3c). For example, upon activation by WNT ligands, Frizzled is internalized in an AP2-dependent manner[18], leading to its degradation, and perturbation of this process induces aberrant embryonic development[18].

However, by promoting the internalization of ligand-bound receptors to endosomes, which can serve as internal signalling platforms, clathrin-mediated endocytosis also activates or amplifies signalling[106,108]. Moreover, under conditions of low ligand concentrations of some RTKs, such as EGFR, clathrin-mediated endocytosis directs the receptors to the endosomal recycling pathway, which allows sustained signalling by

trafficking the receptors back to the cell surface[106,108,110] (FIG. 3c). When the capacity of clathrin-mediated endocytosis is exceeded, clathrin-independent pathways take over and route most of the receptor population for degradation[106,110].

Synaptic vesicle recycling. Synapses are at the core of integration and communication between different areas of the brain, where electrical signals converge to be converted into chemical signals that are transmitted to surrounding neurons. Chemical signals in the form of neurotransmitters are packaged into vesicles for Ca^{2+}-dependent exocytosis[111]. Very active pre-synaptic terminals can contain thousands of vesicles, and there is a need for efficient and local recycling of synaptic vesicle components following exocytosis[112] (FIG. 3d) for successive rounds of signal transmission.

Clathrin-mediated endocytosis is essential for synaptic vesicle recycling and has an integral role in regulating the size[98] and composition of synaptic vesicles[25,82]. The importance of clathrin-mediated endocytosis is highlighted by the synaptic phenotypes of *C. elegans* and *D. melanogaster* that are mutated for components of this pathway[90,113], from studies in which clathrin-mediated endocytosis is perturbed[114,115] and from the abundance of clathrin–AP2-coated vesicles in brain tissue extracts[116]. The high concentration of clathrin components in the brain compared with other tissues, as well as the presence of brain-specific isoforms of adaptor proteins (AP180, epsin 1, amphiphsyin 1 and dynamin 1) and additional splice forms (usually longer splice variants, for example, of EPS15 and amphiphysin 2) is likely to be related to the more specialized use of clathrin-mediated endocytosis in synapses. Phosphorylation is thought to have an important role in regulating clathrin components in synapses and may even be a mechanistic requirement for endocytosis in secretory cells. Indeed, clathrin components are dephosphorylated upon calcium entry[56,117], a process that is tightly coupled to vesicle exocytosis and vesicle endocytosis[118,119]. For endocytic proteins, dephosphorylation in general leads to enhanced protein–protein interactions, promoting endocytosis.

It is often assumed that clathrin-mediated endocytosis in synapses is faster than in other cell types. However, there are two complications here. First, in synapses one cannot readily follow clathrin as a marker for individual events. Therefore, results in synapses are based on population analyses (which may include many different pathways, some of which might be clathrin independent). Second, the time constant for the slow component of endocytosis is not inconsistent with the values for clathrin arrival and disappearance in non-neuronal cells (epithelial cells or fibroblasts), although it is slightly faster[76,120]. However, clathrin-coated vesicles observed in synapses are significantly smaller than other vesicles (FIG. 3a, lamprey synapse) and thus probably take less time to form. In synapses, clathrin-mediated endocytosis is specially coupled to the exocytic stimulus and is therefore specifically tailored to the recycling of synaptic vesicle components (for example, SNAREs and neurotransmitter transporters) rather than to the uptake of ligands.

Exploitation by toxins, viruses and bacteria. Clathrin-mediated endocytosis can be exploited by pathogens, such as toxins, bacteria and viruses. Bacterial toxins can easily be accommodated in clathrin-coated vesicles owing to their small size; for example, anthrax, one of the larger toxins entering through clathrin-mediated endocytosis, is ~25 nm × 10 nm, which is roughly the size of an LDL particle in complex with its receptor. Botulinum toxin B enters synapses by binding to synaptotagmin[121], a cargo for clathrin-mediated endocytosis[17] that is exposed on the cell surface after synaptic vesicle exocytosis. Although tetanus, shiga, diphtheria and anthrax toxins can use clathrin-mediated endocytosis to enter cells[122–125], most of them can also enter cells using other, clathrin-independent pathways[126–129]. For example, the entry of anthrax toxin does not depend on the core clathrin components, AP2 and EPS15 (REF. 130), which is indicative of a clathrin-independent entry route. In fact, the evidence for a clathrin–AP1 endocytic route for anthrax uptake[130] might be the result of a potential decrease in the levels of anthrax receptor on the plasma membrane as a consequence of perturbation of Golgi export or endosome recycling. These effects may have induced by RNA interference of clathrin or AP1 (REFS 131,132).

Bigger cargoes, such as viruses, may require a larger vesicle or may need to change the normal geometry of clathrin-coated vesicle. As an indication, transferrin bound to its receptor has a diameter of ~13 nm (FIG. 3c), and one LDL particle has a diameter of ~25 nm[103]; these cargoes can easily be accommodated in clathrin-coated vesicles with average internal diameters of ~35–42 nm[81,82] without changing their normal geometry. However, among the viruses that are known to enter cells using clathrin-mediated endocytosis, only small viruses, such as rhinovirus or Semliki forest virus have a size (~30 nm in diameter) and shape (spherical) that can fit a clathrin-coated vesicle without altering its formation. Nevertheless, larger spherical viruses, such as reovirus (~85 nm in diameter) and influenza A (~120 nm in diameter) are taken up into clathrin-coated vesicles that must increase their size to accommodate the cargo[76,133]. By contrast, the non-spherical (70 nm × 70 nm × 200 nm 'bullet-shaped') vesicular somatitis virus (VSV) enters cells through endocytic carriers that are composed of a partial clathrin coat around the tip of the virus and require local actin assembly to complete internalization[69]. It is the non-spherical shape of the cargo (combined with its large size) that physically hampers the closure of the clathrin coat and triggers the engagement of the actin machinery, as a 75-nm-diameter spherical mutant of VSV is internalized into classical clathrin-coated vesicles in the absence of the actin machinery[134]. Similar actin-dependent internalization of large (up to 1 μm diameter) clathrin planar structures that are not clathrin-coated vesicles was reported at the bottom surface of adherent cells[29], perhaps reminiscent of the processes mediating some focal adhesion or tight junction internalizations[132,135].

Bacteria and fungi are even larger than viruses, but some (for example, *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersinia pestis,* uropathogenic *Escherichia coli* (UPEC) and *Candida albicans*) recruit

Neurotransmitters
Endogenous molecules that transmit signals from a neuron to a target cell across a synapse.

Exocytosis
The process by which the content of secretory vesicles (such as a synaptic vesicle) is released out of the cell.

clathrin to their sites of entry and depend on clathrin for endocytosis[71,136–138]. These pathogens frequently bind receptors that are known to be internalized in a clathrin-dependent manner. However, considering the size of bacteria (~0.5 μm × 0.5 μm × 0.5–2 μm), it is very unlikely that even their tip can be coated in a clathrin coat in a similar way to VSV. This is consistent with the absence, so far, of electron micrographs showing a clathrin lattice surrounding sites of bacterial entry. However, it should be noted that several clathrin-coated pits can be observed on the surface of cells underneath latex beads (a model system for studying pathogen phagocytosis) and underneath *L. monocytogenes* (FIG. 3b); the creation of these pits might be caused by the clustering of cellular receptors that is induced by these very large cargoes.

Unlike for UPEC and *C. albicans*, AP2 is not required for the entry of *L. monocytogenes*[136]. Instead, PtdIns(4)P (which is present at the TGN) and AP1 (the TGN-localized clathrin adaptor protein) are both detected at the site of *L. monocytogenes* entry, and AP1 is required for the internalization of bacteria[139]. This suggests an alternative mode of clathrin recruitment (and perhaps function) to that used during clathrin-mediated endocytosis (which is totally dependent on AP2) (BOX 1). It is possible that clathrin forms a flat array at the site of pathogen entry (as it does on endosomes[131]) and serves as a signalling platform for actin polymerization, which may thus make it indispensible for cellular entry of these very large cargoes. Alternatively, clathrin might be brought to this site on AP1-positive vesicles derived from the TGN and fuse at sites of *L. monocytogenes* entry, as has been proposed to happen during Fc receptor-mediated phagocytosis[140].

Involvement in human diseases

The loss of function of any of the central components of clathrin-mediated endocytosis — clathrin, AP2, epsin and dynamin — results in embryonic lethality[34,141,142]. As a result, severe mutations of key players are not expected in human diseases. Nonetheless, several perturbations of clathrin-mediated endocytosis proteins have been reported in numerous human disorders, such as cancer, myopathies, neuropathies, metabolic and genetic syndromes, and psychiatric and neurodegenerative diseases (see Supplementary information S1 (table)).

Cancer. Many proteins involved in clathrin-mediated endocytosis have been reported to be perturbed in human cancers (see Supplementary information S1 (table)). Translocations and fusions of genes coding for clathrin components (such as clathrin heavy chain, clathrin assembly lymphoid myeloid leukaemia (CALM), endophilin, EPS15 and huntingtin-interacting protein 1 (HIP1)) with transcription factors or kinases in lymphomas and leukaemias were the first evidence of the involvement of clathrin-mediated endocytosis in cancer[143,144]. However, it is still not clear whether the pathology arises from the impairment of clathrin-mediated endocytosis or from impairment in the function of the fusion partners. Only a few proteins involved in clathrin-mediated endocytosis have been reported to have their expression levels changed in tumours (see Supplementary information S1 (table)). This could be because overexpression, or underexpression, of most accessory proteins involved in clathrin-mediated endocytosis induces endocytic defects *in vitro*[55]. It is interesting to note that the changes in protein expression levels always seem to be similar across tumours. For example, the expression of DAB2 and Numb is downregulated in all of the six cancer types to which these proteins are linked, probably leading to the accumulation of specific receptors on the cell surface, and HIP1-related (HIP1R) and cortactin expression levels were increased in their respective tumours. This could suggest that only a few perturbations in the levels of proteins involved in clathrin-mediated endocytosis lead to an 'advantage' that supports oncogenic transformation.

Systematic characterization of somatic mutations in cancer genomes have identified mutations in clathrin and several of its associated proteins and adaptors (EPS15, HIP1, CALM, endophilin and β-arrestin 1) in breast, renal and lung cancers[145,146]. Whether these mutations are directly involved in the pathology is not known yet. Notably, somatic mutations and alterations in the expression of genes encoding clathrin components were found mostly in solid tumours, whereas fusion genes have been found so far exclusively in blood cancers, such as leukaemias and lymphomas. This raises the possibility that dividing cells (which form solid tumours) can survive only when perturbations in clathrin-mediated endocytosis are mild, whereas fully differentiated cells (such as immune and other blood cells) can tolerate more marked perturbations. Consistent with this, inhibition of clathrin-mediated endocytosis has virtually no consequences in B cells[147,148], whereas it induces cell cycle perturbations and multiploidy in epithelial cells[148–150].

Myopathy, neuropathy, psychiatric and neurodegenerative diseases. Mutations in amphiphysin and dynamin have been reported in forms of myopathy (for example, centronuclear myopathy) and neuropathy (for example, Charcot–Marie–Tooth disease)[151–153]. The link between the consequences of the mutations and the development of the disease is still unclear. In addition, it is possible that these mutations do not affect clathrin-mediated endocytosis, as dynamin functions in clathrin-independent endocytic pathways (BOX 1) and the amphiphysin isoform used in muscle tissue does not bind to clathrin or AP2 and is required for muscle structure[154]. Mutations, single nucleotide polymorphisms and altered expression of several genes encoding clathrin-mediated endocytosis proteins have been reported in patients with psychiatric disorders (such as bipolar disorder and schizophrenia) and neurodegenerative disorders (for example, Alzheimer's disease) (see Supplementary information S1 (table)), but no direct links between the gene perturbations and disease onset or development have been firmly established.

Metabolic and genetic syndromes. Mutations in the cargo-specific adaptor protein ARH and in SH2 domain-containing inositol phosphatase 2 (SHIP2) have been reported in autosomal recessive hypercholesterolaemia and type 2 diabetes, respectively[58,155]. In the case of ARH, the mutations have been established to cause

Fc receptor
A surface molecule found on various cells that binds to the crystallizable fragment (Fc) regions of immunoglobulins, thereby initiating immune effector functions.

Somatic mutations
Mutations, or change in genomic sequence, happening in somatic cells (thus, non-inheritable).

Multiploidy
An abnormal number of chromosomes (> 2n), usually the result of defective cell division.

Single nucleotide polymorphisms
DNA sequence variations occurring when a single nucleotide differs between paired chromosomes in an individual or between members of a biological species.

the disease by disrupting its role as an LDLR-specific clathrin adaptor protein. Furthermore, the genes encoding synaptojanin 1, intersectin 1 and dual-specificity Tyr-phosphorylation-regulated kinase 1 (DYRK1A), which are located on chromosome 21, are duplicated in patients with Down's syndrome[156,157]. The predicted increased expression of these genes has been confirmed, but the contribution of the protein overexpression in the syndrome's pathology remains uncertain.

Conclusion and perspectives

Although the importance of clathrin-mediated endocytosis is unchallenged, its role in various physiological responses remains largely unclear. The precise role of clathrin-mediated endocytosis in signal transduction has yet to be fully understood. For example, how does receptor endocytosis lead to the activation of signalling pathways, and how does clathrin-mediated endocytosis induce this? A better understanding is required of the initial steps in signal transduction and of the accessory modules that are associated with clathrin-mediated endocytosis. Perhaps the next stage of understanding of the pathway should indeed focus generally on accessory modules. This will be facilitated by the advent of better inhibitors and a more complete understanding of mechanistic details of the pathway.

The recent development of new technologies, such as gene editing[158] and super-resolution live-cell imaging[159], opens the exciting perspective of studying the endogenous proteins involved in clathrin-coated vesicle formation with unprecedented spatiotemporal resolution.

Many proteins are assumed to be involved in clathrin-mediated endocytosis just by virtue of binding to some of the clathrin components or because they localize to clathrin-coated pits. However, in many cases there is limited or no mechanistic detail supporting their involvement. Given the expanding variety of alternative endocytic pathways, it may well be that some of these associations are imprecise and they may act in different pathways.

Although the core components of clathrin-mediated endocytosis are not mutated in many diseases, there is evidence that mutation of accessory components may have a role in some diseases and in disease progression. Given that late-onset diseases often seem to be responsive to environmental cues, which in turn are responsive to the surface receptor composition of cells, it is likely that manipulations of this pathway will have a significant role in their progression. For example, because disease proteins pass from cell to cell during the progression of aggregation diseases, such as Alzheimer's disease or Huntington's disease, upregulation of clathrin-mediated endocytosis may promote the degradation of unwanted material by the cell before it is transmitted. Thus, there should be some focus on how to specifically upregulate the pathway, and how to target unwanted material in this direction.

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Competing interests statement
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